# The Honeybee as a model to study Worker policing, Epigenetics, and Ageing

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# Preface

*There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.*

*There is another theory which states that this has already happened.*

Douglas Adams, *The Restaurant at the End of the Universe*

This thesis is centred around policing behaviour in the European honeybee, *Apis mellifera* LINNAEUS, 1758. Most of the work described in this booklet has been carried out at the KU Leuven, Belgium, yet some parts of the research have been conducted at the University of Sussex, UK, and at Ghent University, Belgium, in close collaboration with colleagues of the aforementioned institutions.

In the first part of this thesis, I will sketch a short overview over the studied organism, then describe the peculiar behaviour that stands central in this field of research. I'll continue to characterise the standing problems that await solution, before defining my research questions and the aim of this thesis.

In the second chapter, I will focus on a more mechanistic aspect of policing behaviour, namely how bees are able to discriminate worker-laid eggs from queen-laid eggs. I discuss why there hasn't been much progress on this topic despite quite some efforts in the past 20 years or so, and describe my experiments that shed new light on the question how honeybees recognize eggs. I suggest that peptides on the egg surface, and not cuticular hydrocarbons, mark the difference between eggs laid by queens and workers. In doing so, I also emphasize that the focus on apolar compounds, such as cuticular hydrocarbons and esters, might have been impedimental to the discovery of more semiochemicals.

The third chapter deals with the behavioural part of policing behaviour, and is based on observations of honey bee workers removing eggs. I investigate whether there is evidence for specialisation, and report that indeed specialisation occurs between patrilines, indicating that there is a genetic component to policing behaviour. We did not find evidence for a correlation between age and policing behaviour, yet cannot conclude that age polyethism did not apply to worker policing.

Inspired by the epigenetic mechanisms in the honeybee that are involved in caste differentiation, we became interested in the wider role of epigenetics in insects. Chapter four gives an overview over the emerging field of epigenetics in locusts. This chapter has been published in The Journal of Experimental Biology (Ernst *et al.* 2015).

Based on interchange of ideas with my colleagues on the intriguing question of ageing and ageing theories, and diverse views on how to study them, I suggest and discuss in chapter five different avenues in ageing research, as published in AGE (Ernst *et al.* 2014).

In the sixth and last chapter, I will summarise my findings on policing in honeybees, and place them into context of the field of behavioural ecology. I identify gaps in our knowledge and promising avenues for future research. I also shortly address the future implications of the chapters four and five.

This thesis is complemented with a list of conference presentations and invited talks, my publication list, a list of abbreviations and figures, an abstract, and the most read part of any thesis (Cardoen 2011), the acknowledgments.

Chapter one

# 1 General Introduction

## *1.1 Honeybees*

### 1.1.1 Life history

The life history of honeybees has been reviewed many times, *e.g*. Seeley 1985, Winston 1987, Ruttner 1988, Tautz 2007, Tautz 2008, Oldroyd and Wongsiri 2009.

There are seven to twelve extant species of honeybees, depending on the taxonomic authority (Engel 1999, Lo *et al.* 2010). They all form perennial colonies living in and on self-made wax combs, containing generally one queen, several thousands up to 50,000 female workers (the queen's daughters), and during spring and summer several hundreds of males. Most species are found in Asia, with the notable exception of the Western honey bee, *Apis mellifera* LINNAEUS, 1758, which occurred naturally in Africa and Europe, before it was spread all over the world during the past few hundred years due to its suitability for beekeeping.

As all bees (Hymenoptera, Apoidea), honeybees are phytophagous[1], feeding on pollen and sugary solutions, mainly nectar from floral and extrafloral sources, but also sugary secretions from aphids and scale insects, or from rotten fruit. Liquids are imbibed by using their tongue, and transported within the stomach, while pollen is brushed out of their hair and collected in a specialised structure on the tibia of their hind leg, the so-called "pollen basket". Foragers return to their colony, where they unload their freight. The collected food is used to build stocks to survive winter when foraging is impossible, to maintain the workers, and to raise brood. Honeybees are highly eusocial (Batra 1966, Michener 1969, Wilson 1971, Wilson 1975), characterised by reproductive division of labour, cooperative broodcare, and overlapping generations (for a controversial discussion see also Crespi and Yanega 1995, Sherman *et al.* 1995, Costa and Fitzgerald 1996, Reeve *et al.* 1996, Wcislo 1997, Costa and Fitzgerald 2005, Crespi 2005, Lacey and

[1] The exception proves the rule: three species of stingless bees (*Trigona crassipes*, *T. hypogea*, *T. necrophaga*) are actually feeding on carcasses (Roubik 1982, Camargo and Roubik 1991, Noll *et al.* 1996, Noll 1997), and other bees may collect salt, water, and organic material for nesting material (Baumgartner and Roubik 1989). Even honeybees have been observed to forage on blood (Chance 1983) and carcasses (Crewe 1985).

Sherman 2005, Wcislo 2005). Eusociality is a hallmark of evolution and one of the major transitions in evolution (Maynard Smith and Szathmáry 1995, Bourke 2011).

One of the most intriguing features of honeybees is their abstract (partly non-chemical) communication system which they use to indicate lucrative foraging sites. Successful foragers may indicate the direction (relative to the position of the sun) and the distance of a food source by the famous waggle dance (von Frisch 1923, von Frisch 1946, Haldane 1955, von Frisch 1965, von Frisch 1967, Aristotle 1991, Brockmann and Sen Sarma 2009, Grüter and Farina 2009b, Grüter and Farina 2009a, but see also Wenner and Wells 1990, Tania 2005). The same communication system is also employed when colonies swarm and decide to which new nest site they should move (Seeley 2010).

Within the true honeybees (*Apis*), the European (or Western) honey bee, *Apis mellifera* LINNAEUS, 1758, forms some the largest colonies with up to 40,000 female workers at their peak in development, and during summer times a few hundred to thousands of drones (males).

Throughout this thesis, the terms "bee" and "honeybee" refer to the European honeybee, *Apis mellifera* LINNAEUS, 1758, and not the whole genus *Apis* of the true honeybees. More strictly, most reported experiments have been done on the European subspecies *A. m. mellifera, A. m. carnica, A. m. ligustica,* and breeds thereof. Therefore, results should not be generalized to other species, and even between subspecies substantial biological differences occur that prevent general conclusions (Ruttner 1988). The most intriguing differences are found in *A. m. capensis*, where workers are able to reproduce by thelytoky (Onions 1912, Goudie and Oldroyd 2014), with interesting implications for the study of policing (*e.g.* Pirk *et al.* 2003). Other differences may occur in the speed of policing and replacement of removed eggs (Kärcher and Ratnieks 2014) or the onset of worker laying after queen loss (Ruttner and Hesse 1981).

### 1.1.2 History: of men and bee

The honey bee is the most beneficial insect to mankind. Its relation with humans has been reviewed extensively by Crane 1983 and 1999. In earliest (pre-historic) times it has been valued for its honey production, and likely also the brood has been consumed. The oldest evidence for humans robbing honey is a cave painting dating back at least from 6000 BCE (Crane 1999). Later, humans succeeded in keeping bees in man-made structures. The oldest texts and pictures demonstrating beekeeping have been found in Egypt. To date, the oldest archaeological remnants of an apiary have been found in Israel ($10^{th}$ – $9^{th}$ centuries BCE) (Bloch *et al.* 2010). Beekeeping has also been described in ancient Indian and Chinese documents. It was common practice in the Greek and Roman societies, as well as in Germanic and Celtic cultures. In medieval times, Charlemagne

obliged farmers to keep bees. However, beekeeping relied on relatively crude methods for a long time. Modern beekeeping became only possible after the development of beeswax comb foundation by Mehring and Schober and the centrifugal extractor for honey by von Hruschka, and the important insights and improvements of Dzierzon, von Berlepsch and Langstroth, who propagated the movable frame that have enabled us to manipulate bee hives without destroying their combs (the movable frame had been invented on many occasions, maybe already over 2000 years ago, but had failed to spread (Herold and Weiß 1999)).

For the cultural history of honeybees see Glock 1891, Dutli 2010, Dutli 2012 or some references in Hogue 1987.

### 1.1.3 Economy

*Honeybees are sweetness and light – producers of honey and beeswax – so it is no great wonder that humans have prized these small creatures since ancient times.*

Thomas D. Seeley, *Honeybee Democracy*

The economic importance of honeybees stems not so much from the products of bees (mainly honey, pollen, wax, royal jelly, propolis, venom) as from its pollination service (van Engelsdorp and Meixner 2010, but see also Breeze *et al.* 2011, Aebi *et al.* 2012, Ollerton *et al.* 2012). Long before the discovery that many flowers need to be pollinated by insects (Sprengel 1793), European honeybees have been exported to the Americas, and later to Australia and New Zealand (Crane 1975, Crane 1999, van Engelsdorp and Meixner 2010). In Europe, some commercial and hobby beekeepers move their colonies to rapeseed fields or to fruit trees. In the Unites States of America, millions of colonies are moved around the continent for pollination services for *e.g.* almond, apple, cherry, and plum (California), alfalfa, clover, and sunflowers (North Dakota, South Dakota), blueberry (Michigan), cranberry (Wisconsin), watermelons, pumpkin, and cucumber (Texas), and Brazilian pepper (Florida) (Morse and Calderone 2000, Rosner 2013). The value of pollination by honeybees has been estimated to be 14.6 billion US$ annually for the USA alone (Morse and Calderone 2000); for Belgium, it has been calculated to be 472 million US$ (316 million €) (Simoens *et al.* 2003). The global economic value of this pollination service has been estimated by different authors to be 179 billion US$ (Simoens *et al.* 2003), 117 billion US$ (Costanza *et al.* 1997), 200 billion US$ (Pimentel *et al.* 1997), 190 billion US$ (153 billion €) (Gallai *et al.* 2009), and 360 billion US$ (Lautenbach *et al.* 2012). While these numbers are difficult to compare as they use different assumptions, models, and corrections for inflation, they all underline that pollination by honeybees is of substantial economical value, not to mention the ecological value.

### 1.1.4 General scientific interest

*I've never met an animal, or a plant for that matter, that wasn't interesting, but some stand out as special. Cichlid fishes are right up there.*

George Barlow, The Cichlid Fishes: Nature's Grand Experiment In Evolution

Judging based on this quote, it is evident that Barlow 2000 has never studied honeybees, which have been called a "pinnacle of social evolution" (Wilson 1975). Honeybees have been described by Greek and Roman authors, however most of their descriptions are mythical. Aristotle likely described the waggle dance (Haldane 1955, Aristotle 1991), but other stories are that far from reality that it is sometimes hard to believe these authors ever observed bees closely. The scientific study of honeybees began with Swammerdam (1637-1680) who studied anatomy, physiology, and behaviour of social insects (Cobb 2002); the earliest image produced by light microscopy also depicted bees (Stelluti 1625 *fide* Cobb 2002). Important advances were then made by Réaumur and the famous blind entomologist Huber in the 18th century, and by Dzierzon in the 19th century (Maeterlinck 1901).

The honeybee is of special interest to science, and it was the fifth insect genome (Robinson *et al.* 2006) that had been sequenced (at a time where genome sequencing and annotation was still in its infancy, *i.e.* the "previous generation") (Honey Bee Genome Sequencing Consortium 2002, Honey Bee Genome Sequencing Consortium 2005, Weinstock *et al.* 2006, Wilson 2006). It has been suggested that the study of the honeybee will be beneficial to human health, as honeybees are excellent models for immunology as well as sources for discovery of novel antibiotics. Several components of bee venom have "outstanding therapeutic potential for cancer, sleep disorders, learning and memory enhancement, Parkinson's disease, HIV and AIDS associated dementia, schizophrenia, and novel non-viral vector development for gene therapy" (Honey Bee Genome Sequencing Consortium 2002).

They are also used as models for social behaviour and underlying genetic traits (eusociality), self organization (emergence, division of labour), communication (abstract symbolic "language", *i.e.* waggle dance), ageing (queens live several years, but workers only several weeks (Page Jr and Peng 2001)), learning (bees can be readily trained to show whether they had learned something), cognition (bees have cognitive maps for navigation, and master abstract concepts like "sameness" (Giurfa *et al.* 2001)), and odour perception (which can be studied simultaneously at the level of cells and brain substructures).

### 1.1.5 Development and epigenetics

It is intriguing to note that from any fertilized egg, either a worker or a queen phenotype may arise (polyphenism). The developmental pathway towards a queen phenotype can be activated even after three days of development as a worker, indicating that this decision is not genetic but based on the food the young larvae receive (Weaver 1966, Haydak 1970, Winston 1987, Page 2013). Honeybee workers (nurse bees) nourish the larvae by glandular secretions of their mandibular gland and hypopharyngeal gland (Patel *et al.* 1960, Jung-Hoffmann 1966, Haydak 1970, Beetsma 1979), and potentially also by secretions from the postcerebral gland and thoracic gland (Fujita *et al.* 2010, Fujita *et al.* 2013). They also add nectar or honey and pollen as appropriate. Thus, nursed are in control of the developmental fate of their sisters and can either castrate the larvae (to produce workers) or boost them (to produce queens) (Page 2013). The details of this intricate feeding scheme are not yet completely elucidated (Page 2013). The glycoprotein royalactin, an important part of the larval food, is necessary to induce the queen phenotype via activation of the Epidermal Growth Factor Receptor (EGFR), and induces also in *Drosophila melanogaster* larger, longer-lived, and more fertile individuals with shorter development time (Kamakura 2011). We showed that royalactin delays ageing also in *Caenorhabditis elegans* (MAUPAS, 1900), which is for several reasons a valuable model organism to further dissect the mechanism of how royalactin exerts its beneficial actions (Detienne *et al.* 2014). Eventually, these processes lead to higher titers of Juvenile Hormone (JH) and ecdysteroids (Rachinsky and Engels 1995, Hartfelder 2000, Leimar *et al.* 2012), and to differential methylation of DNA (Wang *et al.* 2006, Kucharski *et al.* 2008, Foret *et al.* 2009, Lyko *et al.* 2010, Foret *et al.* 2012). In fact, down-regulation of DNA methyltransferase 3 (Dnmt3) induces queen phenotypes (Kucharski *et al.* 2008). Differential methylation in turn seems to produce differential splicing variants that may control alternative developmental pathways (Foret *et al.* 2009, Foret *et al.* 2012). Interestingly, microRNAs seem to act complementary to DNA methylation in caste determination (Ashby *et al.* 2016).

It has been hypothesised that conflict between paternal and maternal genome leads to differential methylation between patrigenes and matrigenes (*i.e.* genes inherited from father and mother, respectively) (Haig 2002, Queller 2003, Johnson and Linksvayer 2010, Drewell *et al.* 2012), which might lead to parent-specific gene expression, as shown in mice (Gregg *et al.* 2010a, Gregg *et al.* 2010b). From a sociogenomic point of view, it would be most interesting to study this in honeybees and other social insects where conflict and cooperation have been intensively investigated. Currently, such studies are being conducted on bumblebees (Jelle Van Zweden, personal communication) and honeybees (Kocher *et al.* 2015, Galbraith *et al.* 2016).

We have recently proposed a possible connection between epigenetics and the function of hormones ("epi-endocrinology") (De Loof *et al.* 2013). The involvement of epigenetics in another insect polyphenism is discussed in chapter 4, Epigenetics and locust life phase transitions (Ernst *et al.* 2015).

## *1.2 Policing*

### 1.2.1 Conflicts within a superorganism

It is nowadays widely recognized that the eusocial insect societies, despite their appearance as "superorganisms" (Wheeler 1911, Seeley 1989, Moritz and Southwick 1992, Hölldobler and Wilson 2009), harbour several actual or potential conflicts, *e.g.* over sex allocation, reproductive allocation, caste fate, and male parentage (reviewed in Ratnieks *et al.* 2006, Bourke 2011). As generally in life, these conflicts are all about reproduction (Ratnieks and Reeve 1992). This is expected because the members of a honeybee colony are not genetically identical (see Figure 1). Therefore, different parties may have different interests concerning the production of female and male sexuals. Because honeybee workers are not able to mate (reviewed in Butler 1956), they cannot fertilize their eggs. Hence, they are generally unable to produce females, because this requires heterozygosity at the complimentary sex determination (CSD) locus (reviewed in Heimpel and de Boer 2008). As an exception to the rule, one subspecies of honeybees, the Cape honeybee *Apis mellifera capensis* ESCHSCHOLTZ, 1822, is able to produce females by thelytokous parthenogenesis (thelytoky) (Onions 1912, reviewed in Rabeling and Kronauer 2013, Goudie and Oldroyd 2014). There are also reports that virgin queens and laying workers of European subspecies are occasionally able of producing ca. 1 % female offspring (Mackensen 1943, Tucker 1958), but this seems extraordinarily seldom. In the USA a line of thelytokous honeybee workers has been selected where worker-laid eggs (WLE) develop into workers and queens (DeGrandi-Hoffman *et al.* 1991), but they are likely of African descent (Morris-Olson 2002) and might therefore bear elements of Cape honeybees rather than representing thelytokous European races.

In general however, workers are not able to produce females, and therefore the queen is not challenged when it comes to the production of females[2], unless it is in the interest of workers to not produce females, or not to produce sexuals at all.

However, because honeybee workers, as workers in many other social insects (Wilson 1971, Bourke 1988, Choe 1988), are capable of laying viable haploid (unfertilized) eggs by arrhenotokous parthenogenesis (arrhenotoky) that will give rise to males (Dzierzon 1845, Dzierzon 1898), there is potential conflict about the production of drones.

As shown in Figure 1, any worker is related[3] by 0.5 to her own son (Trivers and Hare 1976, Bourke 1997). Due to haplo-diploidy, all workers in a colony are related by 0.25 to their brothers (the sons of their mother, the queen). Workers are related by 0.375 to their nephews, the sons of their full sisters (a female sired by the same mother and father). Finally, workers are related by 0.125 to the sons of their half-sisters (a female sired by the same mother but a different father). Because the mother queen is multiply mated in honeybees, the majority of the females in a colony are half-sisters. Therefore, a random worker is related with a random son of another worker by 0.15 on average (assuming an effective paternity of 10). *Ceteris paribus*, a worker should always prefer to raise her own sons (r=0.5). If given the choice, she should prefer her nephews (r=0.375) over her brothers (r=0.25). The least favourable option would be to raise "half-nephews", *i.e.* the sons of her half-sisters (r=0.125).

Based on these numbers, it can be (and has been) predicted that workers have an incentive to lay male eggs (which they often do), that they should prefer their own eggs and these of full sisters over the queen's male eggs (which they do), and that they should not be in favour of the male offspring of half-sisters (which has also been observed). Based on these numbers, it should be (and has been) surprising that many social insects

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[2] Even when workers are able to produce females, they are more closely related to full-sisters (daughters of the queen, r=0.75) than to their own daughters (r=0.5). Therefore, as long as the effective paternity is not larger than two, the female offspring of the mother queen should be preferred. This is also true for the thelytokous offspring of a sister and for daughters of a sister mated with a brother (r=0.625 for single mating). These somewhat complicated patterns can be found in Ratnieks 1988.

[3] Throughout this thesis, the reported relatedness values are „life-for-life relatedness". These are "regression relatedness" values (Grafen 1985) weighted by a sex-specific reproductive value (assuming the absence of worker reproduction, which is not completely met, yet does not alter the values dramatically); explanations can be found in Grafen 1986, Grafen 1991, Crozier and Pamilo 1996, Bourke 1997, one of the more accessible ones is Bourke and Franks 1995, and just how confusing these and other relatedness measurements can be is exemplified by Crozier and Pamilo 1980.

workers refrain from laying male eggs in the presence of the queen, in contrast to our prediction. Exactly these calculations were the basis for the discovery of policing (see paragraph 1.2.3, Discovery of worker policing).

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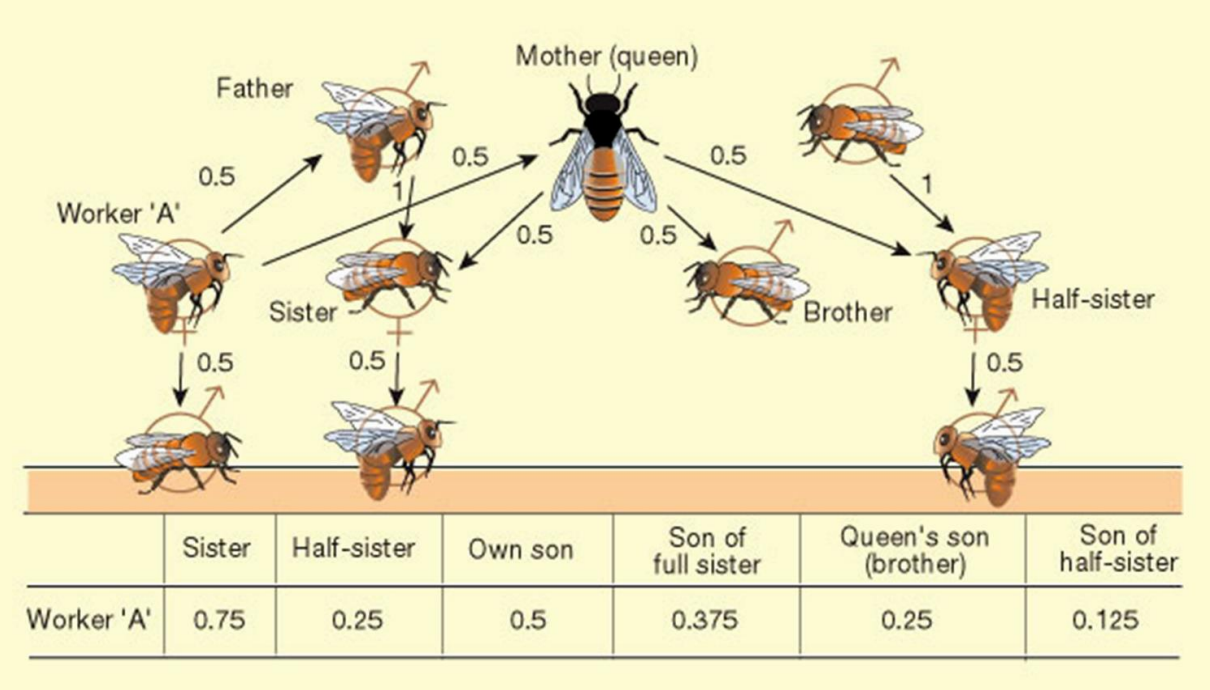


| | Sister | Half-sister | Own son | Son of full sister | Queen's son (brother) | Son of half-sister |
|---|---|---|---|---|---|---|
| Worker 'A' | 0.75 | 0.25 | 0.5 | 0.375 | 0.25 | 0.125 |

***Figure 1 – (Life-for-life) Relatedness within a honeybee colony.*** *Modified and reproduced with kind permission of Whitfield 2002.*

On basis of these observations, it has been suggested that mechanisms must be in place that help to resolve this conflict about male parentage, namely policing (see paragraph 1.2.2, Conflict resolution and policing in social insects).

Alas, things in real live tend to get messier than presented in the textbooks. For instance, a special situation arises when the colony decides to swarm with the mother queen. Not only is there conflict about how often a colony should swarm (Visscher 1993).

Because the new queen is a daughter of the mother queen, and therefore only related by 0.25 to most of the remaining workers, they tend to "rebel" against the new queen (their half-sister) (Nieh 2012, Woyciechowski and Kuszewska 2012). After all, the workers then are only related by 0.125 to the sons and daughters of the new queen (neglecting the contribution of the full sisters in this simplified calculation). Another further complication is that the relatedness values are based on the assumption that there is no worker reproduction and no inbreeding, whereas at least the former is not met (Bourke 1988).

However, this does not affect the general applicability of our hypotheses that is further developed in the following paragraphs. Differing views are discussed in section 1.2.4.7, Environmental and ecological factors.

### 1.2.2 Conflict resolution and policing in social insects

Conflict resolution by policing is widespread in social insects and has been observed in at least 29 and 36 species (worker and queen policing, respectively), all in the order of Hymenoptera (Ratnieks *et al.* 2006). Based on relatedness, worker policing is also expected for termites "unless supplemental reproductives mate with sibs" (Ratnieks 1990b). There is, however, little evidence for worker policing in termites (Hoffmann and Korb 2011). Worker policing has been defined as "any behavior, or possibly physiological attribute, by one worker that acts to bias the production of males away from other workers and in favor of the queen" (Ratnieks 1988), or slightly modified as "any action by one worker which reduces direct reproduction by other workers" (Ratnieks 1990b). Later, this definition had been broadened to also include "coercive actions that reduce direct reproduction by other individuals" (Monnin and Ratnieks 2001), however, at the expense that policing would then be more difficult to discern from reproductive competition.

Oldroyd lists no less than eight types of policing alone for the social insects (Oldroyd 2013), and Monnin and Ratnieks 2001 give additional examples. Simplified, policing can be categorized by the actor (queen, worker, gamergate in the case of some queenless ants (Monnin *et al.* 2002), or self (Ratnieks 1988)) and by the means (brood removal, antagonism toward fertile workers, prevention of egg laying[4], selective trophallaxis (Ratnieks 1988), and mutilation (Monnin and Ratnieks 2001, Monnin *et al.* 2002)).

[4] Oldroyd *et al*. 1999 suggest that yet another type of policing exists, where brood pheromone emitted by larvae prevents the development of ovaries. While it is true that worker bees do respond to brood pheromone which effectively prevents ovary activation (Jay 1970, Kropacova and Haslbachova 1970, Kropacova and Haslbachova 1971, Arnold *et al*. 1994, Mohammedi *et al.* 1998, Winston and Slessor 1998,

**Queen policing** has been observed mostly in smaller insect societies, where the queen is able to control most brood and interact with many of the workers (for some examples see Bonckaert *et al.* 2011a). It is considered to be less efficient than worker policing, because one single individual (the queen) is competing with potentially many "outlaw" workers (Ratnieks 1988, Bourke 1999). In se, queen policing is a form of selfish policing by the queen (see below). Whether multiple mating is a strategy of queens to induce worker policing in larger societies, as has been suggested by Starr 1984, is doubted by others (Ratnieks 1988).

**Gamergate policing** would be a special case of queen policing in ants that do not have queen castes, where the dominant alpha (gamergate) specialises on reproduction (Monnin and Ratnieks 2001). In some cases, gamergates can actively trigger worker policing (Monnin *et al.* 2002).

**Worker policing** is common in *Apis*, as well as in many ant and wasp species. It is regarded as the most efficient policing strategy, because potentially all workers could be involved in policing each other.

**Self-policing** (self-constraint, acquiescence) is thought to occur when selfish behaviour would be so costly at the colony level that the inclusive fitness of an individual would be reduced, and therefore is selected against, *i.e.* individuals do not attempt to reproduce (Cole 1986, Ratnieks 1988, Pamilo 1991b, Wenseleers *et al.* 2004a). For honeybees, the costs of worker reproduction are likely too small to select alone for reproductive self-restriction (Visscher 1989).

**Egg policing** is the most common strategy applied by both queens and workers and consists in the destruction of eggs, usually by eating them. Policing could also occur at later brood stages and in theory also result in the killing of adult males, but the recognition of worker produced males and male brood is likely impossible, and the costs associated with such behaviour are regarded as too high (Nonacs and Carlin 1990, Nonacs 1993).

**Physical policing** or **policing by aggression** has been suggested as a means to prevent worker reproduction. Aggression towards fertile individuals (*i.e.* with activated ovaries) have been observed in honeybees (Sakagami 1954, Velthuis 1976, Schneider and McNally 1991, van der Blom 1991, Visscher and Dukas 1995, Dampney *et al.* 2002, Malka

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Maisonnasse *et al*. 2009, Maisonnasse *et al.* 2010b, Traynor *et al*. 2014), this cannot be regarded as policing behaviour, because worker bees "choose" to react to these signals (Keller and Nonacs 1993).

*et al.* 2008[5,6]) and ants (Hölldobler and Carlin 1989, Crosland 1990, Gobin *et al.* 1999, Kikuta and Tsuji 1999, Liebig *et al.* 1999, Monnin and Ratnieks 2001, Iwanishi *et al.* 2003, Stroeymeyt *et al.* 2007, van Zweden *et al.* 2007, Smith *et al.* 2009, Schmid *et al.* 2013, Teseo *et al.* 2013); in wasps, I am only aware of one study on the prairie yellowjacket *Vespula atropilosa*, where one fertile worker from a queenless colony was killed when the colony was reunified with the queenright part (Landolt *et al.* 1977[7]). However, only in the case of the Indian jumping ant *Harpegnathos saltator* it was actually shown that aggression effectively lead to the regression of ovaries (Liebig *et al.* 1999), whereas the other studies provided merely observations of aggressive interactions[8]. In honeybees it seems unlikely that policing by aggression is of major importance, as aggression towards fertile bees was not always higher than aggression toward non-fertile workers (Visscher and Dukas 1995, Dampney *et al.* 2002[9]), and especially given the highly efficient egg removal behaviour (Visscher 1996). However, aggression during an attempt to oviposit

[5] Barron and Oldroyd 2001 cite also Evers and Seeley 1986 as an example of aggression towards fertile workers, yet the latter have not investigated this; rather, they show that in queenless colony consisting of only two patrilines, half-sisters are a little more often aggressed than full sisters, but this tendency was very weak. Evers and Seeley suggest that this aggression might be interpreted as selfish, as it might increase the chances of laying eggs themselves. Note that this case of kin-discrimination has been doubted by Hogendoorn and Velthuis 1988 and Moritz and Heisler 1992 who found that bees fail to discriminate between half- and fullsisters when several patrilines are present; however, Visscher 1998 maintains that kin discrimination is real, and explains the mentioned diverting results by their lack of statistical power.

[6] Ratnieks 1988 cites Anderson 1963, but the latter does not provide data that corroborate this claim.

[7] Kikuta and Tsuji 1999 cite Akre *et al.* 1976 as an example of aggression toward fertile workers in *Vespula atropilosa*; however, Akre and colleagues 1976 only suggest to investigate ovary status. Landolt *et al.* 1977 eventually did this experiment.

[8] Brunner *et al.* 2009b observed that ovaries regressed when queenless colonies were reunified with a queenright colony and suggest that this was due to prolonged physical policing; however, they could not exclude other effects, including the presence of the queen. Malka *et al.* 2007 show that for honeybee workers, fertility and sterility are fully revertible, and that it is most likely the effect of queen and brood pheromone, and not policing or self-restraint, that lead to the (re)establishment of worker sterility. Also in the buff-tailed bumblebee, *Bombus terrestris*, reversion to fertility is possible under direct queen-influence in the pre-competition point phase (Alaux *et al.* 2007), but again this was not due to physical worker policing.

[9] However, the experimental design in the study of Dampney and colleagues suggests that they observed aggression as a consequence of colony reunification rather than because of ovary activation, which might explain their mixed results (in some colonies, bees with activated ovaries received more, in other less aggression, and the trends for anarchistic and wild type bees were sometimes in the same, but sometimes in the opposite direction).

may be successful in some cases, as observed in the tree wasp *Dolichovespula sylvestris* (Wenseleers *et al.* 2005).

A special case of aggression is represented by mutilation of subordinates to prevent their development into gamergates that could challenge the reproductive monopoly of the current gamergate (Monnin and Ratnieks 2001). In some cases, aggressive policing may lead to the death of the policed individual (Landolt *et al.* 1977, Gobin *et al.* 1999, Monnin *et al.* 2002, Schmid *et al.* 2013, Teseo *et al.* 2013). Deadly aggression has also been reported for honeybees (Anderson 1963[10]).

**Selfish policing** ("corrupted policing" (Wenseleers *et al.* 2005)), not to be confused with self-policing (see above), is a form of policing where the reproduction of other workers is prevented to increase the chances of reproduction of the policing individual instead of the queen, contrary to the original definition of policing given by Ratnieks 1988. It is regarded to be identical to dominance behaviour (Bourke 2007). Therefore, the term "selfish policing" has been criticised by some for mixing the concepts of policing and reproductive competition. However, in contrast to competition, it is targeted specifically at WLE and/or reproductive workers, thereby increasing the queen's reproductive output (Bonckaert *et al.* 2011a). An early observation of this phenomenon in the prairie yellowjacket *Vespula atropilosa* is reported by Landolt *et al.* 1977, and more examples for this behaviour can be found in paper wasps (Saigo and Tsuchida 2004, Liebig *et al.* 2005), vespine wasps (Wenseleers *et al.* 2005, Bonckaert *et al.* 2011a[11]), two ants (Stroeymeyt *et al.* 2007, Brunner and Heinze 2009, Brunner *et al.* 2009a), and arguably bumblebees (Zanette *et al.* 2012).

Policing, coercion, punishment, spite, dominance, sanction, competition, sabotage, etc. are concepts that are often overlapping yet never congruent; some of these terms are discussed by Clutton-Brock and Parker 1995, Reeve and Keller 1997, Monnin and Ratnieks 2001, Raihani *et al.* 2012, Singh and Boomsma 2015. *E.g.*, policing by aggression

[10] Although it has not been observed directly, but rather dead bees have been collected, of which there were more than usual after queen removal.

[11] However, Wenseleers and colleagues did not test whether egg-laying workers did police more than sterile workers.

can be regarded as punishment[12] *sensu* Clutton-Brock and Parker 1995 if it reduces the propensity of the aggressed individual to reproduce (or to become a reproductive (in the case of caste conflict or to become a gamergate)), but egg eating is not, because this does not hinder the egg-eater to lay more eggs (Reeve and Keller 1997, Monnin and Ratnieks 2001). The latter could be regarded as spite when performed by workers (Foster *et al.* 2000, Foster *et al.* 2001, Gardner and West 2004; see Gardner and West 2006 for an easily accessible introduction to the concept of spite).

***Figure 2 - Distrust and social control in honeybees.*** *Reproduced with kind permission of Parkins in Okasha 2010.*

### 1.2.3 Discovery of worker policing

In 1929, Verlaine suggested that some workers in queenright colonies lay eggs and sire some of the male offspring (Verlaine 1929 *fide* Ribbands 1953), yet this seemed not very plausible, given that no fertile workers were found by dissections, and no new brood is seen when the queen is removed. Gontarski noted in 1938 that workers lay eggs in normal queen-right colonies, and predicted that they would remove worker-laid eggs

[12] By the definition of Clutton-Brock and Parker, punishment requires that the punished individuals change their behaviour afterwards. However, the requirement of a change in behaviour may lead to counterintuitive situations where the act of punishment does not qualify as punishment sensu Clutton-Brock and Parker. Imagine an individual does not respond to the punishment, *e.g.* continues to act selfish, maybe because the costs of being punished are low compared to the gains, or because the risk of being punished is relatively small due to detection failure of offence. In this situation, punishment would not be effective, and the act of punishment would be qualified as "spite" (Foster *et al.* 2000, Foster *et al.* 2001). On the other hand, it could be argued that ineffective punishment does qualify as "proper" punishment as long as it yields, on average, the desired effects.

(WLE) in the brood nest (Gontarski 1938). In the late 1970s and early 1980s, Hamilton's interpretation of natural selection (Hamilton 1963, Hamilton 1964b, Hamilton 1964a, Hamilton 1972) became increasingly more popular (*e.g.* Dawkins 1976, Dawkins 1989, Grafen 2004), and students of social insects started to apply these then new concepts to their own field (*e.g.* Trivers and Hare 1976, Alexander and Sherman 1977). Christopher Starr discussed the consequences of multiple mating on patterns of relatedness within a colony and was the first to note that under multiple mating, workers should "police each other's reproduction" (Starr 1984). The same was noticed in a book on honeybees without using the word "policing" (Seeley 1985). Different interpretations have been made by Woyciechowski 1985 who suggested that workers should refrain from laying eggs if the queen is multiply mated; however only in Polish which at that time and likely for the next decennia (centuries?) will not be of prime importance in the scientific literature. The same author then presented his hypothesis formally in a mathematical model (Woyciechowski and Lomnicki 1987). A year later, the first formal model for the evolution of policing was presented (Ratnieks 1988). The first experimental evidence for worker policing then came from Ratnieks and Visscher 1989. It is interesting to note that all of these early discussions of worker policing are based on honeybees. Also of interest is that the phenomenon of policing had been predicted based on simple models (not much more than a table with relatedness values for different relations within a family, see Figure 1), which allowed predictions that could be tested experimentally.

### 1.2.4 Explanations for the occurrence of worker policing

Here I discuss several current hypotheses that have been brought forward to explain the existence of worker policing in social insects. Various mutual non-exclusive scenarios may account for the evolution of policing behaviour in social insects, and have to deal with the idiosyncrasies of queen versus worker policing on the one hand, and policing by aggression versus policing by egg eating on the other hand. While queen policing is generally to be expected, the occurrence of worker policing is not always easy to understand. In section 1.2.5, Origin and evolution of policing in insects, I discuss possible routes for its evolution, and in section 1.2.6, Maintenance of policing behaviour, I discuss the maintenance of policing.

#### 1.2.4.1 Relatedness hypothesis

The relatedness hypothesis states that the reduced relatedness with WLE in colonies with a multiply mated queen should lead to worker policing, as workers thereby gain a relatedness advantage (see paragraph 1.2.1, Conflicts within a superorganism). This was the original hypothesis and led to the study of policing in social insects in the first place

(see paragraph 1.2.3, Discovery of worker policing). All things being equal, any worker in a colony would be selected to remove eggs by other workers if the effective queen mating frequency was higher than two. By and large this prediction has been experimentally confirmed for a sufficient number of social insects of diverse taxa (Wenseleers *et al.* 2004b, Wenseleers and Ratnieks 2006a). Nevertheless, worker policing has been observed in a number of species where, based on relatedness alone, no worker policing would be expected (*e.g.* in clonal ants (Hartmann *et al.* 2003, Brunner *et al.* 2009a, Teseo *et al.* 2013), in a parthenogenetic subspecies of the honeybee, the Cape honeybee *Apis mellifera capensis* ESCHSCHOLTZ, 1822 (Pirk *et al.* 2003), or societies of wasps, ants, bumblebees, and hornets with effective paternity less than two (Kikuta and Tsuji 1999, Foster and Ratnieks 2001a, Foster *et al.* 2002, Iwanishi *et al.* 2003, Endler *et al.* 2004, Saigo and Tsuchida 2004, Wenseleers *et al.* 2005, Zanette *et al.* 2012, Schmid *et al.* 2013). Yet, this does not refute the relatedness hypothesis, since all things may not be equal. Indeed, from the beginning, also colony efficiency has been considered to be an important factor (see 1.2.4.2, (Group productivity) cost hypothesis (= colony-level efficiency) which is not mutually exclusive with relatedness arguments (Ratnieks 1988, Bourke and Franks 1995, Frank 1996, Hammond and Keller 2004).

Occasionally, the role of relatedness seems to have been overemphasized, likely because it is an appealing idea and relatedness is relatively easy to assess in comparison to colony level productivity (Bourke 2011). Therefore, several authors caution not to overemphasise the relevance of relatedness (West Eberhard 1975, West *et al.* 2007) by neglecting ecological factors, proximate constraints (Bonckaert *et al.* 2011b), and costs and benefits (Beekman 2004, Moore and Liebig 2010).

Yet, experimentally induced reduction in relatedness increased efficiency of policing by aggression towards fertile workers in the ant *Temnothorax unifasciatus* (Walter *et al.* 2011), further supporting the relevance of the relatedness hypothesis.

#### 1.2.4.2 (Group productivity) cost hypothesis (= colony-level efficiency hypothesis)

It has been argued that worker reproduction should be policed if it imposed too high costs at the colony level (*i.e.* a trade-off between selfish direct fitness and altruistic indirect fitness) (West Eberhard 1975, Cole 1986, Ratnieks 1988). Worker reproduction might be costly if reproductive workers work less, consume more food, engage in costly dominance interactions, produce less viable brood, produce too many males, or if the production of sexuals occurs too early in the colony life cycle (thereby reducing the overall production of sexuals) (see also 1.2.4.3, Adaptive reproduction schedule hypothesis). Another cost would occur when workers destroy by accident queen-laid eggs (QLE) (Ratnieks and Reeve 1992, Nonacs 1993).

In the extreme, these costs might lead to self-policing (self-restraint (Ratnieks 1988), acquiescence (Wenseleers *et al.* 2004a, Wenseleers *et al.* 2004b, Wenseleers and Ratnieks 2006b); but see Holmes *et al.* 2014 who suggest that only highly efficient policing but not relatedness will lead to acquiescence.

The cost hypothesis is often invoked when the predictions of the relatedness hypothesis are not met. Indeed, there are several reports suggesting that reproductive workers work less (reviewed in Wenseleers *et al.* 2004b). However, the evidence that worker reproduction is costly at the colony level is scarce (Bourke 2011). Two studies could not detect costs of worker reproduction (Lopez-Vaamonde *et al.* 2003, Dijkstra and Boomsma 2007), another two found only little costs (Cole 1986, Gobin *et al.* 2003). In the ant *Diacamma* sp., the occurrence of laying workers reduced the longevity of non-reproductive workers, but did not affect the short-term colony brood production (Tsuji *et al.* 2012). Recently, it has been suggested that policing in the clonal raider ant, *Cerapachys biroi*, is aimed at colony efficiency (Oldroyd 2013, Teseo *et al.* 2013), but they did not test this experimentally. The costs of reproducing of a few workers in a large colony are likely negligible (Wenseleers *et al.* 2004a). Lately, a mathematical model has suggested that the costs incurred by adjusting the sex ratio or by replacing worker-destined brood by males (who do not work) are sufficient to explain the occurrence of policing in the absence of relatedness benefits (Wenseleers *et al.* 2013).

#### 1.2.4.3 Adaptive reproduction schedule hypothesis

Recently, another hypothesis has been put forward by Ohtsuki and Tsuji 2009 who show in a mathematical model that worker policing may also evolve at an ancestral state (single mating, one queen) in some conditions as a consequence of ergonomic benefits at the colony level (adaptive reproduction schedule hypothesis). Therefore, it may be seen as a variation on the cost hypothesis (see above). In short, workers in a small colony should prefer[13] to invest in growth first (ergonomic phase, raising new workers) and starting investment in reproduction only when the colony reaches a certain size (reproductive phase, raising sexuals), because in total this allows to produce more sexuals (*i.e.* increased inclusive fitness). Therefore they should do both, self-restrain and police each other strongly during the ergonomic phase, and reproduce and police selfishly (reproductive competition, dominance behaviour) yet less vigorous during the reproductive phase.

[13] When talking about "strategies" and "preferences", I do not mean to invoke that individuals or genes actually do have strategies or preferences but that it appears as if they had (see for instance Dawkins 1976, Dawkins 1979).

Once such a behaviour has been established, it could be further employed in colonies with higher mating frequencies, where it might have led to the evolution of "true" or genuine worker policing (*i.e.* not selfish). This model is supported by data in wasps (Bonckaert *et al.* 2011b), bumblebees (Duchateau and Velthuis 1988), and ants (Moore and Liebig 2010, Walter *et al.* 2011, Moore and Liebig 2013).

#### 1.2.4.4 Viability hypothesis for egg policing

It had been suggested that WLE are less viable than QLE (Velthuis *et al.* 2002, Pirk *et al.* 2004), and that this would be sufficient to explain worker policing (Gadagkar 2004). Indeed, WLE seem to be more sensitive to desiccation when kept at low humidity (Velthuis *et al.* 2002, Wegener *et al.* 2010). In contrast, one study finds no difference in viability (Ratnieks and Visscher 1989), and another even reports higher viability of WLE (Beekman and Oldroyd 2005). My own data do not indicate any differences in hatchability between WLE and QLE (chapter 2, Peptides mark the difference between eggs of queens and workers in honey bees). It may well be that the high humidity usually employed in laboratory experiments conceals potential differences in hatchability (Wegener *et al.* 2010). However, honeybees do not seem to be able to identify dead embryos (eggs), as they are not removed (Beekman and Oldroyd 2005, Martin *et al.* 2005b, Kärcher and Ratnieks 2014); this suggests that it is not viability per se that is used as a cue to remove eggs. In the common wasp *Vespula vulgaris,* WLE are less viable than QLE, but the authors conclude that this alone does not explain worker policing (Helanterä *et al.* 2006). In the buff-tailed bumblebee *Bombus terrestris*, no difference in viability between QLE and WLE exists, and yet are WLE policed by both queen and workers (Zanette *et al.* 2012).

If drones reared from WLE had a reduced fitness compared to drones reared from QLE, this again might select for policing of WLE. However, there is no data indicating that drones reared from WLE are less fit. Smaller drones (*i.e.* reared in the smaller worker cells) are indeed inferior to larger drones (Berg 1992, Berg *et al.* 1997, Gencer and Firatli 2005, Couvillon *et al.* 2010), but workers lay preferentially in drone cells (Gontarski 1938, Free and Williams 1974, Page and Metcalf 1984). The effect of egg maternity (QLE vs. WLE) has not yet been investigated.

Note that this does not rule out viability as an ultimate reason for egg policing. Indeed, if WLE were significantly less viable, it would seem advantageous to remove them even in the absence of relatedness differences (Nonacs 2006). This might happen in a way similar to the removal of diploid drone larva in honeybees that are recognized at early larval stages, likely by a changed cuticular hydrocarbon (CHC) profile (Santomauro *et al.* 2004, Herrmann *et al.* 2005), and removed (Woyke 1963). The removal of diploid drone larva conveys considerable savings in investment, since all the food and time invested in

raising the drone and feeding the adult would be wasted in terms of fitness (Ratnieks 1990a). Diploid drones may occur at high frequencies if a queen mates with close relatives, which might explain why the selective pressure for the evolution of a removal signal for diploid drones seems to have been strong enough. In contrast, the costs of not removing a WLE might be relatively small (see paragraph 1.2.9, Costs and benefits and best strategies in egg policing, and Wenseleers *et al*., in preparation). Therefore, evolution of egg policing based purely on viability reasons might be more difficult. While the viability hypothesis receives relatively little attention in the social insect community (but see Velthuis *et al.* 2002, Gadagkar 2004, Pirk *et al.* 2004, Tautz 2008), it is relatively difficult to design experiments to test it. After all, the predictions of the viability hypothesis point in the same direction as the relatedness hypothesis: WLE eggs should be removed, and the earlier the better.

However, if viability and associated costs would be the prime reason to remove eggs, we would expect in a comparative approach a pattern of policing that follows the egg viability of male eggs. In contrast, worker policing is highly negative correlated with relatedness, *i.e.* in colonies with low worker-worker relatedness strong and efficient policing is found (Wenseleers and Ratnieks 2006a). Indeed, Walter and colleagues showed experimentally that reduction in relatedness increased efficiency of policing by aggression towards fertile workers in the ant *Temnothorax unifasciatus (*Walter *et al.* 2011). Additionally, other forms of policing (*e.g.* agression towards fertile workers) would be hard to explain by the viability hypothesis alone.

#### 1.2.4.5 Adjustment of sex ratios hypothesis

Another, less popular hypothesis to explain the occurrence of egg removal by workers is the adjustment of the primary sex ratio (sex allocation (Charnov 1982, West 2009) (Pamilo 1991a, Foster and Ratnieks 2001b, Ratnieks *et al.* 2006). In short, there may be conflict about the relative investment in sexuals, the queen preferring an equal investment in her own sons and daughters because she is equally related to either (Fisher's sex ratio 1:1[14] (Fisher 1930, Edwards 1998)), whereas workers should prefer an investment ratio of three to one at the population level since they are three times more related with (super)sisters (r=0.75) than with brothers (r=0.25) (Trivers and Hare 1976) (although things may get much more complicated than that within a colony, *e.g.* Chapuisat and Keller 1999, Meunier *et al.* 2008). Thus, while the queen may control the primary sex ratio (by laying male and female eggs in the ratio she desires), workers may manipulate

[14] However, Fisher used arguments given verbatim by Darwin 1871 and mathematically by Düsing 1883 (reviewed in Edwards 1998).

the secondary sex ratio by removing undesired eggs or brood. Yet, because of multiple mating which reduces the average relatedness with sisters from r=0.75 to a value close to that of half-sisters (r=0.25), there is no or little conflict about sex allocation between queen and workers in the honeybee (Moritz 1985, Pamilo 1991a, Pamilo 1991b, Ratnieks *et al.* 2006). However, the interaction between kin conflict about male parentage and sex ratios may explain the variation in worker reproduction for similar relatedness structures (Wenseleers *et al.* 2013).

#### 1.2.4.6 Reproductive competition and selfish policing

Occasionally, worker policing is regarded as mere reproductive competition. While this seems a more parsimonious interpretation of egg eating and physical aggression in some instances, especially when selfish policing is involved, this fails to account for the vast majority of cases where policing workers are not fertile themselves. Hypothetically, reproductive competition might have played a role in the evolution of policing (see paragraph 1.2.5.1, Dominance behaviour and selfish policing).

#### 1.2.4.7 Environmental and ecological factors

Other reasons to remove eggs (outside the context of egg policing) are environmental factors, *e.g.* when eggs are laid outside the reproductive season (*i.e.* males are usually only raised in spring and summer) (Boes 2010), when eggs are laid too far away from the main brood area (Francis Ratnieks, personal communication), or when longer periods of bad weather occur (reviewed in Boes 2010). Both queens and workers influence the amount of male eggs and male brood, which results in a complicated regulation of drone production within a honeybee colony (Wharton *et al.* 2007, Wharton *et al.* 2008, Boes 2010).

#### 1.2.4.8 Relevance of the various hypotheses

Most of these hypotheses are not mutually exclusive (Riehl and Frederickson 2016). As most of their predictions are similar and the factors involved difficult to disentangle, it remains challenging to reject some of them. Having said that, the predictive power and empirical support for the combined relatedness and colony efficiency hypothesis make them more useful than the more general hypotheses that are not (yet) supported by mathematical models.

Some authors caution to not use the expression “policing” and argue that the term “oophagy” might be better suited, as it would not be tinted by human experiences (Hunt 2007). This view has been heavily criticized by Ratnieks 2008, who argues that “policing” is a broader concept than egg eating, which is entirely correct.

Other hypotheses are discussed in the following section on the evolution of policing.

### 1.2.5 Origin and evolution of policing in insects

It is quite possible that policing and especially worker policing evolved for relatedness and/or colony efficiency reasons (see above). However, policing could also have evolved for other reasons first and might subsequently have been co-opted and maintained for other reasons, too. Several of these additional hypotheses are discussed below. Recent theoretical work suggests that policing cannot easily evolve, yet it is readily maintained (El Mouden *et al.* 2010) (see paragraphs 1.2.6, Maintenance of policing behaviour, and 1.2.9, Costs and benefits and best strategies in egg policing). The evolutionary origin of policing behaviour has been discussed by several authors (*e.g.* Foster and Ratnieks 2001a, Zanette *et al.* 2012). The behavioural traits involved (*e.g.* aggression, reduced trophalaxis, egg eating) are present in social insects and would not require *de novo* evolution (Ratnieks 1988). Policing requires signalling and recognition systems (to recognize fertile individuals, and to discriminate WLE and QLE). Sophisticated recognition systems are present in social insect colonies and are also used in nest mate recognition, therefore they could be employed in the context of policing. Thus, only signalling would be a new trait. Again, chemical signals might be based on pathways already present, and it seems that fertility cues are directly linked to reproductive physiology in most cases, explaining the cues present on eggs and bodies of fertile individuals (Blomquist and Bagnères 2010). Only in the case of the enigmatic egg marking pheromone in the honeybee, it might be that an additional signal has evolved (which would be to the advantage of both queen and worker, see 1.2.7.3, Egg discrimination by chemical cues or signals - CHCs). This egg marking pheromone seems to be evolutionary conserved (to some degree) between the Western honeybee *A. mellifera* and the dwarf honeybee *A. florea*, which diverged ca. 6 – 10 million year ago, but seems to have further evolved in the Asiatic honeybee *A. cerana* (Nanork *et al.* 2007b). Workers of *A. mellifera* did not differentiate between QLE and WLE of *A. cerana*, and the authors speculate this might be due to the (postulated) ability of *A. cerana* workers to mimic QLE, which would also explain the slow removal of WLE in *A. cerana* colonies (Oldroyd *et al.* 2001).

#### 1.2.5.1 Dominance behaviour and selfish policing

The removal of eggs is common in social insects and also occurs in parasocial insects (semisocial, quasisocial) (Michener 1969, Wilson 1971, Michener 1974). In parasocial insects, individuals may compete for reproduction, *e.g.* in the large carpenter bee, *Xylocopa sulcatipes* (Stark *et al.* 1990), or in association of foundresses in polistine wasps (Gervet 1964, West-Eberhard 1969, Turillazzi and West-Eberhard 1996). This would qualify as dominance behaviour. If queens evolved a trait of egg eating, stemming from reproductive competition, it is likely that subordinate females also express this trait (in

primitive eusocial insects, there is little differentiation between the queen and worker caste).

Alternatively, queens might have evolved egg eating to suppress worker reproduction of their own daughters (basically, queen policing is a special sort of dominance behaviour or selfish egg eating). Again, egg eating by workers might then be a trait originally stemming from queen behaviour.

Yet another possibility is that worker policing behaviour evolved from selfish policing, *i.e.* from reproductive competition amongst workers. Potentially, the selfish component has been lost later, leading to the "classic type of policing" (Bourke 2011). This route has been suggested for the buff-tailed bumblebee, *Bombus terrestris* (Zanette *et al.* 2012). Selfish policing has also been observed in the paper wasps *Polistes chinensis antennalis* (Saigo and Tsuchida 2004) and *P. dominulus* (Liebig *et al.* 2005), the vespine wasps *Dolichovespula sylvestris* (Wenseleers *et al.* 2005), *D. norwegica* (Bonckaert *et al.* 2011a), and *Vespula atropilosa* (Landolt *et al.* 1977), the ant *Temnothorax unifasciatus* (Stroeymeyt *et al.* 2007, Brunner and Heinze 2009). In the clonal ant *Platythyrea punctata*, the most dominant workers carry out most of the policing (as predicted by Frank 1996) and often become reproductive when the current reproductive is removed (Brunner *et al.* 2009a). However, it is not fighting ability or aggression *per se* that may lead to policing (as could be assumed for dominance interactions); for instance, individuals engaged in colony defence are less likely to police, and vice versa (Barth *et al.* 2010).

In these cases, policing and dominance behaviour are difficult to disentangle (Monnin and Ratnieks 2001). It demonstrates however a potential route to the evolution of "true" worker policing: Hypothetically, dominance interactions and competitive oophagy have evolved first, followed by the evolution of multiple mating of the queen, which would have increased the incentive of policing. Finally, highly efficient worker policing may have led to the evolution of "self-policing" ("acquiescence", self-restraint) (Wenseleers *et al.* 2004a, Wenseleers *et al.* 2004b).

In the honeybee, aggression between workers in a queenless colony (after the end of policing behaviour (Miller and Ratnieks 2001)) might be interpreted as reproductive competition (Evers and Seeley 1986).

#### 1.2.5.2 Intraspecific and interspecific parasitism

It has been suggested that policing may have evolved as a defence against (social) parasitism by non-nestmates (Foster *et al.* 2002). In most (flying) social insects, workers occasionally enter a foreign nest ("drifting behaviour" (Butler 1939)); for the crawling species of ants and termites (where only the sexuals are winged), there are few reports about drifting to date (but see Dobata *et al.* 2009, Cheron *et al.* 2011, who also report

social parasitism). At first, this had been regarded as errors in homing and to be maladaptive. Later however, it has been suggested to be an alternative reproductive strategy (Beekman and Oldroyd 2008). Drifting occurs in both, primitive and highly advanced societies, *e.g.* in several sweat bees (Abrams and Eickwort 1981, Soro *et al.* 2009, Ulrich *et al.* 2009). Astonishing high drifting rates have been observed in both vespine wasps (Benaets 2009) and paper wasps (Sumner *et al.* 2007), where both intra- and interspecific parasitism occurs (Akre *et al.* 1976). Also in stingless bees, workers drift to foreign colonies (Alves *et al.* 2009). In several species of bumblebees, drifting behaviour occurs at low frequencies (Birmingham *et al.* 2004, Birmingham and Winston 2004, Lefebvre and Pierre 2007, Blacher *et al.* 2013a, Blacher *et al.* 2013b, O'Connor *et al.* 2013, Zanette *et al.* 2014), and reproductive workers preferentially drift to reproduce in foreign nests (Lopez-Vaamonde *et al.* 2004, Takahashi *et al.* 2010, Blacher *et al.* 2013b). However, Zanette *et al.* 2012 suggest that the actual drifting rates are too small to favour the evolution of worker policing, but that drifting rates might have been higher historically.

Drifting in the Western honeybee has been known for a long time and occurs regularly (Rauschmayer 1928, Free 1958, Pfeiffer and Crailsheim 1998, Neumann *et al.* 2000, Neumann *et al.* 2001), and sometimes up to 63 % of workers do drift (Boylan-Pett and Hoopingarner 1991). Drifting as a strategy does also occur in several other honeybee species (Paar *et al.* 2002, Nanork *et al.* 2005, Härtel *et al.* 2006, Nanork *et al.* 2006, Nanork *et al.* 2007a, Chapman *et al.* 2009b, Chapman *et al.* 2010a, Chapman *et al.* 2010b).

Interestingly, bumblebee nest guards are more aggressive towards fertile non-nestmates (Blacher *et al.* 2013a), which might present an adaptation against drifting behaviour. In the same vein, honeybee workers in queenless colonies are better than queenright[15] colonies at guarding their nest against non-nestmates (Chapman *et al.* 2009a) (queenless nests stop policing (Miller and Ratnieks 2001) and are therefore especially vulnerable for exploitation by social parasites). However, queenright colonies also admit fewer (potentially fertile) workers from queenless colonies, thus also selecting against potential social parasites (Chapman *et al.* 2009a). In contrast, in the Eastern honeybee *A. cerana* (Asian honeybee), queenless colonies are guarding less against drifters than queenright colonies (Chapman *et al.* 2008, Holmes *et al.* 2013b).

While there is so far no evidence for worker drifting in European hornets, *Vespa crabro*, it has been suggested that policing in this species evolved as a defence against

[15] "Queenright" signifies that a queen is present in a colony; "queenless" means there is no queen present in a colony. Note that "queenless" ants usually refers to ant species that lack a queen caste (and where workers can mate).

nest usurpation by queens (Foster *et al.* 2002). In the paper wasp *Polistes biglumis*, queens discriminate against foreign eggs, occasionally destroying their own eggs in the process (Lorenzi and Filippone 2000).

In light of the evidence presented above, it seems quite possible that policing may (also) have evolved in response to the threat of social parasitism (Foster *et al.* 2002). It could be regarded as a second line of defence, in addition to nest guarding at the entrance (Pirk *et al.* 2007b). This would be in parallel with egg recognition in birds as a defence against cuckoos and other brood parasitic birds (Davies 2000, Kilner and Langmore 2011). Indeed, in bumblebees (Alford 1975, Goulson 2010) and (both, solitary and social) wasps (Field 1992, Cervo 2006), obligate and facultative brood parasitic species are common (Jane Brockmann 1993) (and egg dumping seems to occur widespread in insects (Tallamy 2005)). Once evolved, it may also have been applied to worker reproduction in general and maintained even in the absence of the threat by drifters.

However, if policing evolved as a defence against queen parasitism (nest usurpation or egg dumping (O'Connor *et al.* 2013)), one would also expect the recognition of eggs of foreign queens. This has been observed in queenless colonies of European hornets, where more QLE than WLE were removed (in contrast to the "normal" situation in queenright colonies); however, only two queenless colonies were used (Foster *et al.* 2002). Also two subspecies of honeybees, *A. m. scutellata* and *A. m. capensis*, have been reported to discriminate against non-nestmate eggs; overall, the effect of nest-recognition for eggs was more important than caste (QLE vs. WLE) (Pirk *et al.* 2007b). Yet, this result seems not to be a general rule though, and might rather represent an idiosyncrasy of the life-style of these two subspecies, where *A. m. capensis* has specialised to parasitize both subspecies, which might have developed specific defence mechanisms. Additionally, they only used two sources for foreign eggs (one for male, one for female eggs), thereby committing a classic case of pseudoreplication (Hurlbert 1984) (one of the "seven deadly sins in the study of behavior" (Milinski 1997)). It had been suggested that honeybee workers prefer sisters over unrelated individuals for queen rearing when presented a choice of eggs (Visscher 1986), yet another study could not find any effects of relatedness or "nestmateness" (Woyciechowski 1990). Different studies on the subject of kin discrimination and nepotism gave mixed results, and while some maintain that weak nepotism exists (Visscher 1998), this is still discussed controversially (Wenseleers 2007, Boomsma and d'Ettorre 2013).

#### 1.2.5.3 Predictions based on the relatedness hypothesis

If workers could discriminate between eggs of full-sisters and half-sisters, they should remove those of half-sisters, but not those of full-sisters. To date, there is no evidence that

eggs of full- and half-sisters can be differentiated, however this might also be due to the difficulty to obtain WLE of known maternity, and to the best of my knowledge, such experiments have not yet been reported.

If workers were able to detect whether the mother queen is singly mated, they should prefer to raise nephews and destroy male QLE, whereas if the queen is multiply mated, policing should occur (all things being equal). It has been claimed that workers of the Saxon wasp *Dolichovespula saxonica* are able to react to the number of matings of their mother by facultative policing (Foster and Ratnieks 2000), but this has been disputed by Bonckaert *et al.* 2011b who explain the data of Foster and Ratnieks by colony stage (incipient colonies police more and have less worker reproduction than larger colonies (Ohtsuki and Tsuji 2009)). Similar, honeybees also do not stop policing when their mother is (artificially) singly mated (Loope *et al.* 2013), which I had anticipated (see the arguments below).

The predictions above are based on the assumption that workers are able to perceive and to react adaptively to the mating status or the egg maternity, but there are reasons to assume that this is not met (Keller 1997). Theoretically, a worker could get an idea of the number of matings by detecting the variance in cuticular hydrocarbons (CHCs) encountered in other workers (Bonckaert *et al.* 2011b), but it is doubtful whether wasps are able to do so (Dani *et al.* 2004), and also one ant species is limited in its use of CHC information (Boomsma *et al.* 2003). In the Saxon wasp *Dolichovespula saxonica*, this information is not sufficient (Bonckaert *et al.* 2011b). In honeybees, the CHC profile is sufficiently different to discriminate patrilines (the offspring of one father) (Arnold *et al.* 1996), and honeybee workers are indeed able to discriminate half-sisters (Getz and Smith 1983, Getz *et al.* 1986). However, because honeybee queens are very promiscuous (Tarpy *et al.* 2004, Tarpy *et al.* 2010, Tarpy *et al.* 2015), workers will hardly ever encounter a situation where their mother is singly mated, and therefore the chances to evolve such a behaviour (*i.e.* react to differences in variance in CHC profiles) are small. This is in accordance with other behaviours where individual workers in theory would profit by showing nepotistic behaviour (*e.g.* in queen rearing) but fail to do so (reviewed in Keller 1997, Wenseleers 2007).

#### 1.2.5.4 Policing as an adaptive and variable behaviour

Interestingly, policing is not necessarily a strict behaviour that is expressed independent of environmental cues. Honeybees, for instance, police WLE in a queenright colony, both WLE and QLE when initially hopelessly queenless, and stop policing when massed egg laying starts (Miller and Ratnieks 2001). The latter might possibly be initiated by the appearance of esters produced in the workers' Dufour glands (Martin *et al.* 2002b).

Additionally, worker policing might vary with season (Ratnieks 1993, Visscher 1996), and it has been found that worker egg laying is targeted to the swarming season (Perepelova 1929, Woyciechowski and Kuszewska 2012, Holmes *et al.* 2013a). Models predict that colony stage is also important (Ohtsuki and Tsuji 2009), which has been found in several species (see paragraph 1.2.4.3 above).

In addition to temporal effects, the efficiency of policing may differ within a colony based on location within a nest. In the Saxon wasp *Dolichovespula saxonica*, more worker-produced males are raised in small cells on the upper comb, compared to the large cells on the lower comb (Bonckaert *et al.* 2011b). This might be because combined queen and worker policing are more efficient on the larger-celled comb (where the queen spends more time) and/or because workers preferentially lay eggs in the small cells. Honeybee workers preferentially lay eggs in large (drone) cells (Gontarski 1938, Free and Williams 1974), likely because drones reared in small (worker) cells have a reduced fitness (Berg 1992, Berg *et al.* 1997, Gencer and Firatli 2005, Couvillon *et al.* 2010). Interestingly, policing is also faster in drone cells (Halling and Oldroyd 2003), and even more WLE are removed in drone cells (Kärcher and Ratnieks 2014), maybe because eggs are controlled more frequently, or because the acceptance thresholds are higher (Reeve 1989, Sherman *et al.* 1997).

The social parasite *A. m. capensis* seems to avoid the queen and lays preferentially further away from the queen (Neumann *et al.* 2003a). Interestingly, in the "host race" *A. m. scutellata* and in the social parasite, policing further away from the queen was less efficient (Neumann *et al.* 2003a), thus this behaviour seems highly adaptive for the parasite (Neumann *et al.* 2003b). These two subspecies seem to be generally less efficient in policing in comparison to other races, as more queenright colonies contain worker derived males (references within Neumann *et al.* 2003a). Also in *A. m. ligustica*, bees further away from the queen are more likely to act as if queenless, *e.g.* by activating their ovaries and laying eggs (Orlova and Hefetz 2014).

In European hornets, *Vespa crabro*, QLE are retained in queenright colonies, but discriminated against when the queen has been lost (Foster *et al.* 2002); unfortunately, only two queenless colonies have been studied.

Finally, facultative worker policing depending on the mother queen's number of matings has been suggested (Foster and Ratnieks 2000), which would be a strong support for the relatedness hypothesis, yet this report has been doubted (Bonckaert *et al.* 2011b).

### 1.2.6 Maintenance of policing behaviour

The evolution of policing seems to depend on many factors and "may be harder to evolve than previously thought"; yet its maintenance appears to be easier to be

accomplished (El Mouden *et al.* 2010). Once policing behaviour has evolved, it may persist even in clonal animals in the absence of kin conflict, *e.g.* in the clonal ants *Platythyrea punctata* (Hartmann *et al.* 2003) and *Cerapachys biroi* (Oldroyd 2013, Teseo *et al.* 2013). Potentially, this might be regarded as vestigial or atavistic behaviour. However, it is more likely that such policing helps to improve colony fitness (and thus individual fitness) (Ratnieks 1988, Frank 1996), as too many reproductives may reduce the colony efficiency (Hartmann *et al.* 2003, Pirk *et al.* 2003). In the case of *Platythyrea punctata,* policing may also be maintained because of occasional occurring colony fusions and sexual reproduction (Hartmann *et al.* 2005), which would again induce kin conflict (Barth *et al.* 2010). Recently, Wenseleers *et al.* 2013 showed that the combined effects of conflict about male paternity and sex ratio can lead to the evolution of policing even in monandrous colonies.

Whether the trait of worker policing and queen policing will be maintained depends largely on the associated costs and benefits, as discussed in the following paragraph 1.2.9, Costs and benefits and best strategies in egg policing.

### 1.2.7 Identification of worker-laid eggs (WLE)

#### 1.2.7.1 Egg discrimination independent of cues on the egg

Reliable policing, *i.e.* removal of worker-laid eggs (WLE), requires that WLE can be discriminated with a certain confidence from queen-laid eggs (QLE). Theoretically, otherwise identical eggs could be discriminated against if they are deposited in a characteristic way different from QLE, or if a mark (cue or signal) would be left in the vicinity, or if the egg-laying is witnessed by a policing agent. Indeed, honeybee workers in queenless colonies occasionally dump their eggs also on cell walls, whereas a queen normally deposits her eggs on the ground of a cell. However, many WLE are deposited on the ground of cells and seem not to differ from QLE (personal observation) and yet are policed. Also, there was no influence of the number of eggs per cell on policing rates (Katzav-Gozansky *et al.* 2001). There is also no evidence that the queen marks the cells where she lays her eggs (Zeng and Le Conte 2009), and WLE do not survive longer in a cell where the queen had been laying an egg shortly before (Ratnieks and Visscher 1989). Finally, WLE are always recognized by workers when artificially introduced into colonies (Martin *et al.* 2005b). Therefore, the discrimination between QLE and WLE has to be based on differences between these two egg types.

#### 1.2.7.2 Egg discrimination based on physical differences

WLE and QLE may differ in some physical properties, *e.g.* length (which is roughly around 1.5 mm for both), but the available data is somewhat inconsistent (Gontarski 1938, Ratnieks 1993, Woyke 1994, Woyke 1998, Miller and Ratnieks 2001, Katzav-Gozansky *et al.* 2003b, Gencer and Woyke 2006) and references within), which might also be attributed to differences between races, seasons, between individual queens and even within a queen (reviewed in Gencer and Woyke 2006). Also, the eggs change during their development and shrink (Woyke 1998, Gencer and Woyke 2006). Furthermore, eggs laid by laying workers under queen-right conditions might differ from WLE in queenless colonies (Ratnieks 1995), as is the case in the stingless bee *Melipona rufiventris* (Sommeijer *et al.* 1984, Koedam *et al.* 1987). The duration of queenlessness may change the properties of WLE, too (Beekman and Oldroyd 2005, Martin *et al.* 2005a, Wegener and Bienefeld 2009). WLE appear more variable in length, width (Woyke 1994) and weight (Wegener *et al.* 2010) than QLE, but even QLE can vary considerably (DuPraw 1960, Al-Kahtani *et al.* 2013)[16]. Given the variability of the QLE size and shape, which would make a discrimination based on these properties unreliable, it seems unlikely that these parameters are used by honeybee workers.

Because WLE eggs tend to desiccate more readily than QLE (Velthuis *et al.* 2002, Wegener *et al.* 2010), it has been suggested that WLE might be recognised as they desiccate (Wegener *et al.* 2010). While this might contribute to the removal of WLE, this has not been tested yet, and it seems unlikely that this is the sole discriminator between QLE and WLE, because WLE are usually removed within hours (Ratnieks and Visscher 1989, Ratnieks 1995, Visscher 1996, Pirk *et al.* 2004, Beekman and Oldroyd 2005, Martin *et al.* 2005b).

The physical properties of the egg shell do not seem to differ (or are small and likely not perceivable by bees) when investigated with scanning electron microscopy (SEM) (Katzav-Gozansky *et al.* 2003b, Martin *et al.* 2005b). Other physical properties, *e.g.* reflection or electrical charge, have not been investigated, but seem highly unlikely (although both bumblebees and honeybees are able to perceive electrical fields (Clarke *et al.* 2013, Greggers *et al.* 2013)).

The strongest evidence against a physical difference is the observation that WLE are camouflaged when rubbed against (Ratnieks 1992) or placed next to a QLE (Martin *et al.* 2005b), suggesting that the queen signal that conveys protection is transferable and thus likely of chemical nature. Additionally, this also hints that in honeybees, CHCs are not

[16] Not DuPraw 1961, and one egg was reported to be 33 % to 50 % larger than another, not "nearly twofold", as cited by Martin *et al.* 2002b.

involved in this signal, because they are harder to transfer. For instance, the CHC profile of ant eggs sandwiched between other eggs for 45 min had not changed (D'Ettorre *et al.* 2006).

At the same time, these experiments suggest that it is indeed, as predicted, a chemical signature on QLE that protects them rather than a cue on WLE that makes them unacceptable, although it would still be possible that the "protection" signal on QLE overrides a hypothetical "removal" signal on WLE. Eicosenol had been suggested to be a cue by which WLE could be recognized, because it is only found in workers (Martin *et al.* 2004a, Martin and Jones 2004), but it did not make QLE less acceptable (Martin *et al.* 2005b).

#### 1.2.7.3 Egg discrimination by chemical cues or signals - CHCs

Based on theoretical insights, it has been hypothesized that queens should mark their eggs with a queen signal (egg marking pheromone, egg marking signal), a signal that cannot be forged by a worker (Ratnieks 1988, Ratnieks and Visscher 1989, Ratnieks and Reeve 1992, Ratnieks 1993, Ratnieks 1995). In colonies with multiple mating, this would be in the interest of both queen and workers (Seeley 1989, Ratnieks 1995). This is because queens would have sons (r=0.5) instead of grandsons (r=0.25) and workers would raise brothers (r=0.25) rather than half-nephews (r=0.15 on average for WLE, assuming an effective paternity of 10) (Seeley 1985, Keller and Nonacs 1993, Kocher and Grozinger 2011). Therefore, such a queen signal should be relatively easy to evolve (Lloyd 1983, Ratnieks and Visscher 1989). Caste specific gland secretions are well known in the honeybee, *e.g.* queen mandibular pheromone (QMP) from mandibular glands (Slessor *et al.* 1988, Plettner *et al.* 1997), Dufour gland secretions (Katzav-Gozansky *et al.* 1997b), tergal gland secretions (Wossler and Crewe 1999), Koschevnikow glands (Lensky *et al.* 1991, and another as yet enigmatic source of a pheromone found on the head (Slessor *et al.* 1998, Keeling *et al.* 2003).

As predicted, such a signal has been found in the ant *Camponotus floridanus*, where QLE and WLE resemble in their cuticular hydrocarbon (CHC) profile queens and workers, respectively (Endler *et al.* 2004). Note however that the exact signalling compound was not characterised. Many social insect species rely on differences in CHCs to recognize sex, caste, fertility, and nest mates (reviewed in Monnin 2006, Blomquist and Bagnères 2010). Since CHCs are highly variable and allow the discrimination of half-sisters (Arnold *et al.* 1996), they have been suggested as candidates for the egg marking signal. Indeed, CHCs on QLE and WLE differ in the honeybee (Martin *et al.* 2004b), as well as in *e.g.* the buff-tailed bumblebee *Bombus terrestris* (Ayasse *et al.* 1999), the ant *Pachycondyla inversa* (D'Ettorre *et al.* 2004), the European paper wasp *Polistes dominula* (Dapporto *et al.* 2007),

and the common wasp *Vespula vulgaris* (Bonckaert *et al.* 2012). In the latter, two branched alkanes have been identified that partly protect WLE from removal, indicating that they might be (part of) the egg marking signal (Oi *et al.* 2015b). Interestingly, these alkanes act also as queen pheromone in this species (Van Oystaeyen *et al.* 2014).

Also in the honeybee, CHCs are important in signalling in adult honeybees. The amount of CHCs on the cuticle increases after emergence in both workers (Kather *et al.* 2011) and queens (Babis *et al.* 2014), and CHC profiles differ also qualitatively between freshly emerged queens and 10 day old queens, as well as between mated and unmated queens (Babis *et al.* 2014). Pflugfelder and co-workers found that a single (as yet unidentified) CHC is queen-specific, and that this compound is sufficient to initiate stinging behaviour by other queens (Pflugfelder and Koeniger 2003). Furthermore, this signal is conserved between at least four *Apis* species: *A. mellifera, A. cerana, A. koschevnikovi, A. florea* (Pflugfelder *et al.* 2004). Honeybees also use CHCs for nestmate recognition (Dani *et al.* 2005) and potentially to discriminate different task classes (Kather *et al.* 2011).

Based on the results of Endler *et al.* 2004, Beekman *et al.* 2004 suggested that "[…] it could well be that the role of cuticular hydrocarbons as an egg-marking signal has been overlooked in the honey bee.", and Beekman 2004 therefore argued that "[…], the role of cuticular hydrocarbons as the eggmarking signal in honeybees should be reconsidered." However, for the honeybee, the "dogma" of CHCs as universal chemical class of signature does not seem to hold.

First, the CHC profiles of QLE and WLE become similar after ca. 24 h in a colony, but this does neither protect WLE from being policed nor does it increase the removal of QLE (Martin *et al.* 2004b)[17]. Second, unlike the case of *Camponotus floridanus*, where the transfer of CHCs from queens to WLE protected them to some extent from being policed( Endler *et al.* 2004), the transfer of queen Dufour gland extracts (which contain CHCs and esters) does not protect WLE in the honeybee in the long run (Martin *et al.* 2002b). Third, eggs dissected out of ovaries (of both, queens and workers) are policed, despite the similar CHC profile with laid QLE (for the QLE) (Martin *et al.* 2004b). Martin *et al.* (2004b) also review further evidence that honeybees are not able to perceive or actually do not use linear alkanes for discriminatory tasks.

17 Interestingly though, WLE that have survived for 24 h have a much lower removal rate in honeybees (Ratnieks 1993, Kärcher and Ratnieks 2014) and wasps (Wenseleers *et al.* 2005). This might be either because only eggs that have an intrinsic higher survival rate (lower removal rate) are found after 24 h, or because eggs somehow change their chemical profile, maybe by the many waxes from the comb (compare Ratnieks 1995). This has not yet been investigated.

#### 1.2.7.4 Not CHCs, but maybe esters?

In the golden paper wasp *Polistes fuscatus*, the dominant female potentially recognizes eggs laid by subordinate females or non-nestmate individuals by Dufour gland components (Downing 1991). However, subordinate females generally did not remove any egg (but one that was broken), indicating the absence of egg policing by workers. Note that to the best of my knowledge, there is no proof yet that Dufour gland secretions are effectively smeared on eggs during oviposition, 15-26 % of the eggs treated with Dufour gland extracts were not removed, and this pattern holds true only for nests in the preemergence phase (before the first brood hatches) (Downing 1991).

In the honeybee it had been observed that WLE survive initially longer when they are treated with ethanol extracts of the queen's Dufour gland (Ratnieks 1992), when rubbing WLE against the exit of the queen's Dufour gland (Ratnieks 1995), and when applying hexane extracts of the Dufour gland or its ester fraction or synthetic esters (Martin *et al.* 2005b). The content of the Dufour gland differs between queens, sterile and fertile workers (Katzav-Gozansky *et al.* 1997b, Dor *et al.* 2005), which furthers supported the idea that Dufour gland content is used to mark eggs (Oldroyd *et al.* 2002). Katzav-Gozansky *et al.* 2001 reported the occurrence of minute amounts of esters on QLE, of which some (but not all) were also found in the queen's Dufour gland.

In contrast to sterile workers, egg-laying workers (at least in queenless colonies) do produce small amounts of queen-like esters (Katzav-Gozansky *et al.* 1997b), and even more so do anarchistic (Martin *et al.* 2004a) and parasitic Cape honeybees (Martin and Jones 2004). Yet, the eggs of "normal" fertile bees are policed, suggesting that the esters are not protecting eggs (or are not produced in sufficient amounts[18]). Interestingly, WLE originating from colonies that had been queenless for a prolonged period (more than 2 months) were removed at a rate similar to QLE within the first 2 h, but after 20 h were only a little better protected from policing than WLE from colonies that had been queenless for less than 2 months (Martin *et al.* 2005a). This was paralleled by substantial changes in the content of the Dufour gland of laying workers in colonies that have been queenless for several months (their Dufour gland contained 30-58 % of $C_{28}$-$C_{36}$ esters in contrast to 3-4 % of laying workers in short period queenless colonies (Martin *et al.* 2005a)).

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18 This, however, seems unlikely, because the queen lays many times more eggs than her Dufour gland is larger in comparison to workers (Katzav-Gozansky *et al.* 2001): more than 1500 eggs per day with a Dufour gland content of ca. 17 µg of secretions, whereas laying workers lay no more than 50 eggs per day ($< \frac{1}{30}$th of the queen) with a gland content of ca. 3 µg ($< \frac{1}{5}$th of the queen) (Katzav-Gozansky et al. 1997b).

However, neither esters extracted from the queen's Dufour gland nor synthetic esters did protect WLE from being policed when sprayed on the eggs (Katzav-Gozansky *et al.* 2001, Katzav-Gozansky *et al.* 2002), albeit they used relatively low concentrations. Yet, the same results were found when Dufour glands, extracted or artificial esters have been applied directly on WLE, and where the presence of the esters on the eggs was verified by GC-MS (and actually more than natural was applied) (Martin *et al.* 2002b). The eggs treated with esters were initially removed slower (first 2 h), as had been observed by Katzav-Gozansky *et al.* 2001 for another ester, but the effect was gone after 20 h, even though the esters were still present; this is similar to the situation in colonies that have been hopelessly queenless for more than two months (Martin *et al.* 2005a). Low amounts of esters (similar to the quantities found on QLE) had no effect (Martin *et al.* 2002b).

In an interesting mutant, workers reproduce despite the presence of a queen, which has been dubbed anarchistic behaviour (Oldroyd *et al.* 1994, Barron *et al.* 2001, Chaline *et al.* 2002). WLE of anarchistic workers (in a queenright colony) are more acceptable than WLE of "normal" workers (Oldroyd and Ratnieks 2000), and strikingly enough, also more acceptable than WLE of anarchistic workers in a queenless colony (Beekman and Oldroyd 2003). This has partly been attributed to esters on the eggs, and WLE that survived longest had also the highest amounts of esters on their surface (more than QLE) and were laid by workers that had the highest ester content on their setose membrane (Martin *et al.* 2004a). However, QLE still survived better despite having less esters on their surface, suggesting that esters might somehow disguise the identity of WLE, but not mimic the real queen signal. Also parasitic Cape honeybee WLE are almost as acceptable as QLE (Martin *et al.* 2002a); it remains elusive how this is achieved.

It would be interesting to investigate WLE from anarchistic and Cape honeybees for the presence of proteins and peptides. Unfortunately, there are currently no anarchistic colonies maintained on this planet (Madeleine Beekman, personal communication).

While it remains unclear why high amounts of esters delay egg eating, the above described experiments suggest that esters are not used by worker bees to discriminate between QLE and WLE.

Other investigated tissues (queen mandibular gland, queen spermathecal gland, queen and worker setosa (setose or setaceous membrane)) and compounds (eicosenol, aldehydes) are presented in Martin *et al.* 2005b. Worker setosa membrane extracts had a negative effect on QLE persistence, the others did not affect removal rates of QLE or WLE.

#### 1.2.7.5 Evidence for polar compounds

Thus, it seems that neither CHCs, nor esters, nor other hydrophobic compounds are used as egg marking pheromone in the honeybee. This suggests that either something has been overlooked or data misinterpreted, or that the signal is non-polar in nature. For instance, the egg marking pheromone might be a complex signal, as is the case with the queen retinue pheromone which is a mix of at least nine compounds from at least two different exocrine sources (Keeling *et al.* 2003, Keeling *et al.* 2004, Slessor *et al.* 2005, Le Conte and Hefetz 2008). Alternatively, the signal might be of polar nature, as had been proposed earlier (Ratnieks 1995, Martin *et al.* 2005b). QLE rinsed with apolar compounds (hexane, methylenchloride) were less affected by policing than QLE rinsed with polar solvents (ethanol, methanol), although this might also have been due to more damage caused by these solvents (Ratnieks 1995). QLE rinsed with water are quickly policed (Madeleine Beekman, personal communication). In contrast, QLE immersed for 20 minutes in trypsin solution or in alkaline or acidic buffer solutions or in weak acids or bases were not removed faster than untreated QLE. Since trypsin is an enzyme that cuts proteins in smaller pieces, this led the authors to suggest that proteins are likely not involved in egg recognition (Martin *et al.* 2005b). However, this was tested only in 54 QLE and only for 2 h. The speed of policing varies between and within colonies (Ratnieks 1995, Kärcher and Ratnieks 2014), and this could explain why sometimes policing is considerably slower than expected (for instance, colony D2 removed on one day only ca. 20 % of WLE, but the next day ca. 95 %; and survival of QLE varied between 100 % and ca. 65 % in colony D3 (Martin *et al.* 2005b)).

Because the transfer of the queen signal using organic solvents failed, it seems more likely that the signal is a polar compound (Martin *et al.* 2005b). I provide experimental evidence pointing to that direction in chapter 2, Peptides mark the difference between eggs of queens and workers in honey bees.

#### 1.2.7.6 Egg marking signal vs. fertility signalling

It has been suggested that in the social insects in general, queens signal not only their presence, but also their fertility. These signals are expected to be honest signals (Keller and Nonacs 1993, Peeters and Liebig 2009), *i.e.* indices (Maynard Smith and Harper 2003). This has been confirmed in several ant and wasp species, where the CHC profile of fertile workers is altered towards the profile of queens (Heinze *et al.* 2002, Dietemann *et al.* 2003, Smith *et al.* 2008a, Bonckaert *et al.* 2012, van Zweden *et al.* 2014).

However, occasionally the fertility signal may differ between castes, *e.g.* in the common wasp *Vespula vulgaris*, queens differ from workers mainly in 3-methylnonaicosane, whereas fertile workers differ from sterile workers in two other

alkanes and one alkene (Van Oystaeyen *et al.* 2014). In different species, different compounds seem to be involved in this signalling.

When some CHCs are indicator of fertility as seen in many social insects (reviewed in Liebig 2010), and eggs mirror these patterns (Endler *et al.* 2004, Holman *et al.* 2010, Bonckaert *et al.* 2012), then it is possible that there is not such a thing as a "queen signal" that is strictly caste specific, and the difference between a WLE and a QLE would only reflect the fertility of the respective egg layer. Interestingly, this has been found in the Florida carpenter ant, *Camponotus floridanus*, where in larger colonies, QLE originating of queens in incipient colonies (less fertile) are removed just as WLE, because they lack the shorter-chained CHCs that are characteristic of more fertile queens in established colonies (Endler *et al.* 2006). Egg-laying workers do not differ in their CHC profile from sterile workers, suggesting that they are not reaching the high levels of fertility of mature queens (Endler *et al.* 2007), and that the fertility signal might not be gradually changing. Also in other ants, CHCs related with fertility increase in intensity on eggs and/or body when queens (or egg-laying individuals) mature or are more fertile (Peeters *et al.* 1999, Liebig *et al.* 2000, Hannonen *et al.* 2002, Heinze *et al.* 2002).

On the other hand, this means that in incipient colonies, QLE and WLE are indistinguishable, and indeed no policing is observed in incipient colonies of *C. floridanus* (Moore and Liebig 2010). Interestingly, the introduction of QLE from a foreign, highly fertile queen did not induce the removal of the QLE laid by the incipient (less fertile) queen or of WLE.

In sum, this suggests that the "queen signal" is a gradual sign of fertility, and not forgeable (index, Maynard Smith and Harper 2003) because the necessary CHC production is intimately linked with true fertility (Keller and Nonacs 1993). In theory, this could also apply to the honeybee, because the fertility of a queen (laying 1500 and more eggs per day (Buttel-Reepen 1915, Merrill 1924, Nolan 1925)) cannot be reached by a fertile worker (5-10 (Visscher 1996) or 19-32 (Perepelova 1928 cited in Ribbands 1953) eggs per day; up to 200 for *A. m. capensis* workers (Velthuis 1976)). It has been suggested that fertility might be signalled by CHCs on the body (Babis *et al.* 2014), but this has not yet been investigated in fertile workers and mature queens (van Zweden *et al.*, in preparation). Additionally, the situation is further complicated by a complex pheromonal communication system in the honeybee, including various signals originating from at least 15 glands (Free 1987, Winston and Slessor 1998, Slessor *et al.* 2005, Katzav-Gozansky 2006, Le Conte and Hefetz 2008). For instance, in worker bees, fertility is linked with a change in Dufour gland composition (Katzav-Gozansky *et al.* 1997b), whereas dominance is linked with changes in mandibular gland secretions (Dor *et al.* 2005). Indeed, the amount of long-chained esters (which is characteristic for queens

and laying workers, but absent in sterile workers) in the Dufour gland is higher in virgin queens than in newly mated queens, which is again higher than in one year old queens (Katzav-Gozansky *et al.* 1997a), suggesting that esters might (partly) indicate fertility if these amounts correspond to the amount of esters secreted. The Dufour gland extracts of multiply mated queens were more attractive to workers than those of singly mated queens, which were more attractive than these of virgin queens; however, they contained a smaller absolute amount, but a higher proportion of esters (Richard *et al.* 2011). Artificially inseminated queens do have a higher amount of CHCs and esters on their cuticles than virgin queens, but no differences have been detected between inseminations with semen and with saline buffer (Babis *et al.* 2014). It had been suggested that also QMP production is an indicator of fertility in queen honeybees (Winston and Slessor 1992), and the mandibular glands of queens inseminated with a larger volume were more attractive to workers than those inseminated with low volumes or of virgin queens (Richard *et al.* 2007). However, the published data are contradictory (reviewed in Bortolotti and Cecilia 2014); given the complex mixture of compounds, the multiple glandular origin, and still unknown components of the queen pheromones, this is not further surprising. Thus, the nature of the fertility signal and the role of esters in the Dufour gland remains elusive.

In any case, however, differences in Dufour gland content are not reflected in differences on the egg surface, and both CHCs and esters have been ruled out as indicator (Martin *et al.* 2004b, Martin *et al.* 2005a, Martin *et al.* 2005b). Therefore, it might well be that honeybees once more are an exception to the rule, and that they evolved another chemical signal as an egg marking pheromone.

Although worker bees are likely responding to the fertility of their queen (old queens are often superseded (Winston 1987)), the queen may signal her fertility independent of the eggs, *e.g.* by various gland secretions, and also by the amount of brood in the colony (which also emit pheromones (Le Conte *et al.* 1990, Maisonnasse *et al.* 2009, Maisonnasse *et al.* 2010a, Maisonnasse *et al.* 2010b)). Because the signals of her presence are quickly communicated within a colony by "messenger" bees (in less than 2 h) (Seeley 1979, Naumann *et al.* 1991, Naumann *et al.* 1993), additional fertility signalling via eggs, as seen in some ants and likely termites, seems superfluous (Winston and Slessor 1998, Slessor *et al.* 2005, Katzav-Gozansky 2006, Le Conte and Hefetz 2008). Indeed, eggs of virgin queens are not policed (Beekman *et al.* 2004), indicating that the queen signal is independent of her mating status. Also, honeybee queens stop laying eggs for some periods in winter without disturbing the social organization of the colony, supporting the view that eggs do not convey a message of fertility. WLE of anarchistic worker bees, laid in a queenright colony, are usually protected against removal, but not those WLE laid in

a queenless colony, indicating that the egg-marking signal is independent of the physiological mechanisms of egg production (Beekman and Oldroyd 2003) and thus not a fertility signal. This is corroborated by Malka *et al.* 2009 who found that in queenless honeybees, fertility can be experimentally uncoupled from signalling of fertility and dominance. Furthermore, Orlova *et al.* 2013 showed that the fertility signal in workers is influenced by the social system and can be independent from fertility (which casts some doubt on the reliability of the supposed fertility signalling function of esters). While there are differences in the CHC profile between egg-laying dominant females and not-laying subordinate females in the European paper wasp *Polistes dominula* (Sledge *et al.* 2001), only 11,15-dimethyltritriacontane was an indicator of fertility, whereas 17 other compounds were related to dominance status (Dapporto *et al.* 2007). While these differences are reflected by the CHC profile of the eggs( Dapporto *et al.* 2007), which are differentially policed (Liebig *et al.* 2005, Dapporto *et al.* 2010), this demonstrates that there is no universal link between fertility and CHCs.

### 1.2.8 The source of the egg marking signal

Neither the egg marking signal nor a potential source for it have yet been identified. Because eggs dissected from ovaries are policed, but not eggs passing the *bursa copulatrix* (copulatory organ) (Martin *et al.* 2004b), it is likely that the signal is not acquired during oogenesis, but rather during oviposition (Ratnieks 1995). The Dufour gland had been suggested as source of a signal in the golden paper wasp *Polistes fuscatus* (Downing 1991). Occasionally, bright yellow material can be detected in the vagina of *Polistes fuscatus*, resembling the material in the Dufour gland reservoir, making it imaginable that during oviposition, gland products can be applied to eggs (Downing 1991), but see discussion above. The Dufour gland was also a candidate for the honeybee (Ratnieks 1995, Oldroyd *et al.* 2002), because the gland secretions are caste-specific (Katzav-Gozansky *et al.* 1997b) and the opening of the Dufour gland resides in the vaginal wall (Billen 1987). However, the esters and CHCs of queen's Dufour gland did not protect WLE from being removed (Martin *et al.* 2005b), nor does an egg pass the exit of the Dufour gland directly (Martin *et al.* 2005c), making it unlikely to be the source of the egg marking pheromone. Having said that, the results of Martin *et al.* 2004a suggest that Dufour gland content that accumulates on the setose membrane is transferred to eggs during laying. Other potential sources include the median oviduct, the *bursa copulatrix*, the Koschevnikow gland, the spermathecal gland, the sting sheath glands, and the venom gland (Ratnieks 1995, Martin *et al.* 2005b). The Koschevnikow gland does also secrete proteins (in workers) and glycoproteins (in queens) (Lensky *et al.* 1991); it has been hypothesized that they are merely carrier of volatiles, but this has not been further studied. Generally, most

pheromone glands also secrete proteins (Cassier *et al.* 1994 and references within), making it plausible that proteins or peptides may be used as signalling substances. Apolar extractions of the spermathecal gland did not protect WLE, but polar extracts have not yet been tested (Martin *et al.* 2005b). Spermathecal glands are often absent in honeybee workers (Gotoh *et al.* 2013), which would make this gland a good candidate for the source of an unforgeable queen signal, yet it is difficult to imagine how its secretions could be applied to an unfertilized egg during oviposition, since leakage of the spermatheca would likely also result in leakage of sperm and lead to inadvertent fertilization of eggs. The sting sheath glands are primitive exocrine glands that produce alarm pheromone components in workers and likely also secrete proteinacious compounds (Cassier *et al.* 1994); their function in queens remains unexplored. The occasionally mentioned setaceous membrane (setose or setosa membrane in Martin *et al.* 2004a, Martin *et al.* 2005b) is not a gland itself but rather serves as a releaser platform for volatile pheromones (Lensky *et al.* 1995), likely from sting sheath glands and Koschevnikow gland.

Interestingly, rubbing WLE against the queen's sting made them more acceptable (Ratnieks 1995)[19], and venom is used for signalling in the fire ant *Solenopsis invicta* (Klobuchar and Deslippe 2002, Eliyahu *et al.* 2011), the golden paper wasp *Polistes fuscatus* (Post and Jeanne 1983), and other polistine wasps (Post and Jeanne 1984). Therefore, the venom gland may be currently the most promising candidate (see chapter 2, Peptides mark the difference between eggs of queens and workers in honey bees).

### 1.2.9 Costs and benefits and best strategies in egg policing

Policing is only useful if there are eggs or selfish individuals that try to reproduce. The incentive to reproduce may be considerably lowered by policing (Wenseleers *et al.* 2004a), however other factors also influence the decision to reproduce, *i.e.* other costs to worker reproduction (Cole 1986, Ratnieks 1988, Ratnieks and Reeve 1992, Hammond and Keller 2004, Ohtsuki and Tsuji 2009, Moore and Liebig 2010, Wenseleers *et al.* 2013). Here, I consider only the cost and benefits from the perspective of policing workers, assuming that there is an incentive to actual worker egg laying.

[19] Potentially, this might also be due to the long-chained (waxy) esters found in the sting apparatus of queens but not workers (Blum *et al.* 1983); however, only one of these esters (tetradecyl dodecanoate) is also found (at 1 %) in the Dufour gland of queens and laying workers. Unnaturally high amounts of the longer esters of the Dufour gland protected WLE for up to 20 h, but not longer, from being policed (Martin *et al.* 2002b).

All forms of policing require a successful discrimination between desired and undesired traits, *i.e.* the ability to reliably detect and react to undesired features, and the ability to dependably discern desired from undesired features (Reeve 1989, Sherman *et al.* 1997). In the case of policing by egg-eating, this involves the discrimination of undesired (worker-laid) eggs from queen-laid eggs, in the case of policing by aggression towards reproductive workers, this relies on the recognition of fertility in workers (Sakagami 1954, Velthuis 1976, Visscher and Dukas 1995, Dampney *et al.* 2002). As with any discriminatory task, one might expect a trade-off between accuracy and speed in discrimination (compare *e.g.* Chittka *et al.* 2003), and a balance between costs and benefits associated with the corresponding behaviours.

The costs involve the time and energy invested in controlling cells for their content, as well as costs that occur because the time and energy was not invested in more lucrative tasks (opportunity costs). Additional costs involve the accidental removal of queen-laid eggs (Nonacs and Carlin 1990, Nonacs 1993), as well as failure of removing worker-laid eggs. Removal of brood might result in empty cells, which would lead to higher costs in thermoregulation (Ratnieks 1990a) and comb building, because it would require larger comb area for the same amount of brood. (In physical policing, additional costs may be the risk of injury and death during such aggression.)

There may be several benefits. In terms of relatedness, drones sired by the queen are 67 % more valuable than drones sired by workers (at an effective paternity of 10, see paragraph 1.2.1, Conflicts within a superorganism). Additionally, if policing reduces the incentive for worker reproduction (Wenseleers *et al.* 2004a, Wenseleers *et al.* 2004b) or prevents the production of sexuals during colony growth (Ohtsuki and Tsuji 2009), colony efficiency may be considerably increased. Furthermore, individual policing workers might profit from eating eggs as a source of proteins and other nutrients. If selfish policing occurred, they might also profit from another cell where they could oviposit.

I argue that the costs of inspecting cells and controlling egg provenance are fairly low. Firstly, the search time should be rather low, as eggs are deposited in open cells where they are easily accessible (Ratnieks 1995). Honeybee workers constantly inspect cells for many reasons, *e.g.* to consume honey or pollen, to find a place to rest, to clean cells, to feed larvae (Lindauer 1952, Seeley 1982, Kolmes 1985, Kolmes 1986, Seeley and Kolmes 1991, Johnson 2008a), therefore no additional time needs to be invested in inspecting cells (if policing workers also do other tasks inside the hive), provided that the inspection of an egg does not take too long. If workers could distinguish between haploid and diploid eggs, this might help in reducing the time to consider whether an egg should be policed. However, workers seem not to be able to recognize the ploidy of (at least young) eggs

(Oldroyd and Ratnieks 2000)[20]. It has been suggested that workers could reduce the search time if they would control preferentially drone cells (Wattanachaiyingcharoen *et al.* 2002), where workers usually lay their eggs (Gontarski 1938), and indeed policing is faster on drone comb than on worker comb (Halling and Oldroyd 2003). This might indicate that workers control drone cells more frequently (which might be regarded as an adaptation), or that they are less tolerant to WLE on drone comb, but it may as well be that the number of bees per cell is higher on drone comb, because there are less drone cells per area as they are larger than worker cells (Halling and Oldroyd 2003). Secondly, the energy costs for moving around in the hive are negligible in comparison to the energy required for flight. Finally, even when inspecting cells ("patrolling" (Lindauer 1952)) and identification egg maternity is slow, time is not taken from more important tasks, as honeybees spend many hours seemingly idle in the hive (Lindauer 1952). Indeed, a typical forager leaves the hive only ten times a day (Tautz 2007, Tautz 2008). Workers respond to an increase in colony work load by increasing their activity (Tenczar *et al.* 2014). Other motionless workers might be warming brood (Kleinhenz *et al.* 2003), producing wax, or indeed being asleep with their eyes wide open (Kaiser 1988, Klein *et al.* 2014).

A high rate of accidentally removed QLE would impose a relatively high cost (although the egg resources can be recycled); in the paper wasp *Polistes biglumis*, queens discriminate against foreign eggs, yet destroying more than 25 % of their own eggs in the process (Lorenzi and Filippone 2000). Conversely, honeybee workers make very few recognition errors (Ratnieks 1995), and less than 5.7 % of QLE in worker cells, and less than 12.5 % of QLE in drone cells are removed (Kärcher and Ratnieks 2014). Additionally, 74.9 % of the eggs removed are quickly replaced by the queen (Kärcher and Ratnieks 2014). This indicates that the errors in removal are relatively low. Interestingly, after 48 h, relatively more QLE are removed from drone cells (9.6 %) than from worker cells (4.1 %), likely because it is better to play safe and remove an egg when in doubt (increased acceptance threshold (Reeve 1989, Sherman *et al.* 1997)). Larvae were not policed (Ratnieks and Visscher 1989), potentially because they are indistinguishable; the costs of removing a relatively young larva should not be too high. However, this was tested only once with a small number of larvae, and worker-derived larvae survived less then queen-derived larvae, albeit survival was not significantly different.

[20] Yet, the failure of detecting an effect must not be mistaken with evidence for a lack thereof, and Oldroyd and Ratnieks should better have used an equivalence test instead of an ANOVA.

Because new queens are raised in specialised cells (Winston 1987), there is no danger of destroying a female egg that might become a reproductive sexual (in contrast to the situation in ants (Nonacs and Carlin 1990, Nonacs 1993)).

The decision to remove an egg does not have to be taken hastily, as there is nothing to gain from fast action. A honeybee worker not entirely certain about the egg identity might leave the cell, clean her antennae and tongue, before probing the egg again and again. Therefore, the potential trade-off between speed and accuracy should be shifted towards high accuracy.

Even if she decides not removing the egg, it is very likely that the egg in question will be inspected by many more bees, given that the larvae will hatch after about 72 h (Harbo and Bolten 1981, Harbo *et al.* 1981). Ratnieks 1990b shows that WLE may get visited up to 20 times before they are removed, although the majority of WLE is removed within the first 5 visits.

Assuming a WLE would be probed 10 times (once every 7 h), a detection rate of only 37 % per inspection (=63 % chance of survival) would result in a survival rate of 1 % for worker-laid eggs, which is the reported policing rate in honeybees (Ratnieks and Visscher 1989, Ratnieks 1990b, Ratnieks 1993); but see Holmes *et al.* 2013a who found a rate of worker produced drones of up to 6.2 % during swarming season. While it could be argued that there are ten thousands of cells in a colony, which would make it more likely that some WLE are only rarely inspected or even escape detection, there are also thousands if not ten thousands of worker bees that could police eggs.

Given the low costs of inspecting cells and eggs, workers should take their time and remove eggs only after careful inspection. If there is not more than slight evidence for an egg being laid by a worker, it should not be removed, as other workers (with maybe better discriminatory abilities) likely will revisit the egg and might decide then. However, in cases of doubt, an egg should be removed, even if this increases the number of QLE removed erroneously. The here presented arguments are formally analysed with a mathematical model and discussed in more depth in a manuscript in preparation (Wenseleers *et al.*). This predicted pattern has been confirmed experimentally by Kärcher and Ratnieks 2014.

### 1.2.10 Policing outside the social insects

Throughout the living world, conflicts are present- ultimately about reproduction, proximately about territories, food, sex, and parking spaces (Oldroyd 2013). There are several ways of preventing and/or suppressing conflict (Bourke 2011). One of them is coercion, and policing is a particular case thereof (Ratnieks and Reeve 1992, Ratnieks and Wenseleers 2008). Punishment of cheating is thought to generally increase cooperation

(Frank 1995, Frank 1996, Frank 2003, Frank 2009, El Mouden *et al.* 2010, Bourke 2011), but this depends on the specific conditions (Hauser *et al.* 2014). Coercion is found in diverse systems such as bacteria, plants, invertebrate and vertebrate animals (Ratnieks and Wenseleers 2008). Remarkably, it is not limited to intraspecific interactions, but also occurs in interactions between bacteria and plants (legume-rhizobia-mutualism), between plants and insects (obligate pollination mutualism), and between different fish species (cleaner fish mutualism).

Popular and widely cited examples for policing and punishment include soybeans, *Glycine max*, that punish uncooperative bacterial symbionts, *Bradyrhizobium japonicum,* in this legume-rhizobia-mutualism (West *et al.* 2002, Kiers *et al.* 2003, Kiers and van der Heijden 2006, Kiers and Denison 2008), and other mutualisms with arbuscular mycorrhizal fungi where plants can cut carbon rewards if not enough phosphate is provided (Fitter 2006, Kiers and Denison 2008, Mills and Côté 2010). It has also been described in the obligate pollination mutualism between the yucca *Yucca filamentosa* and its yucca moth *Tegeticula yuccasella*, where flowers containing too many moth larvae are aborted (Pellmyr and Huth 1994, Pellmyr 2003), and in the fig tree-fig wasp mutualism, where the rate of cheating (non-pollinating) fig wasps is negatively correlated with the strength of the sanctions (Jandér and Herre 2010). Also in the mutualism between the tree *Glochidion acuminatum* and its pollinator, the moth *Epicephala* sp., sanctions in form of selective abortion of flowers have been reported (Goto *et al.* 2010), and even in the non-obligatory pollination system of the White Campion *Silene latifolia* and the moth *Hadena bicrurissimilar,* fruit abortion occurs (Burkhardt *et al.* 2009). The ant-plant *Cordia nodosa* sanctions its ant symbiont *Allomerus octoarticulatus* when it fails to protect the plant from herbivory by lower growth of the domatia that serve as housing for the ants (Edwards *et al.* 2006).

In fish, coercion has been reported for instance in the daffodil cichlid *Neolamprologus pulcher*, where subordinates that do not help in territory maintenance and defense and brood care are expelled (Balshine-Earn *et al.* 1998). However, Balshine-Earn and colleagues interpret this behaviour not as punishment, but rather as competition for a favourable position in a breeding queue. Alternatively, punishment might not be overt because subordinates appease through increased helping behaviour (Bergmüller and Taborsky 2005).

In the cleanerfish mutualism, punishment of cheating Bluestreak cleaner wrasse, *Labroides dimidiatus*, that eat mucus or tissue of their host does indeed lead to less cheating (at least in a model experiment), and thus is one of the most convincing examples of true punishment in animals (Bshary and Grutter 2002, Bshary and Grutter 2005, Mills and Côté 2010, Raihani *et al.* 2010).

In the rhesus monkey *Macaca mulatta*, individuals who failed to share a food source they had detected are punished (or ""policed"") (Hauser 1992), and in the meerkat *Suricata suricatta,* the dominant suppresses the reproduction of subordinate females (Young *et al.* 2006). However, the former could also be interpreted as competition for food (as "punished" individuals were not more likely to cooperate in the future) (Raihani *et al.* 2012), the latter being an example not for punishment but for enforcing a reproductive monopoly. Beisner and McCowan 2013 also report prosocial policing in rhesus monkeys. von Rohr *et al.* 2012 discuss policing in chimpanzees, *Pan troglodytes*, and cite additional examples of policing in several primate species, including great apes, baboons, and macaques. They define policing as "impartial interventions by third parties in ongoing conflicts" following Flack *et al.* 2005, which is quite different from the original definition given above (Ratnieks 1988, Ratnieks 1990b). Cant and Young 2013 show in a mathematical model that policing by threats (rather than actual aggression) can shape reproductive conflicts, and Cant *et al.* 2014 suggest that policing by threat is involved in synchronizing births in banded mongooses, *Mungos mungo*; however, their data do not show this unequivocally, and the so-called policing might rather be called reproductive competition.

Further examples of punishment and enforcement in, *e.g.*, birds and naked mole rats, are given by Clutton-Brock and Parker 1995 and Ratnieks and Wenseleers 2008. A critical discussion of the different concepts (coercion, punishment, policing, sanction, negative pseudo-reciprocity, mediation, reconciliation, dominance) and of some typical examples is provided by Raihani *et al.* 2012. Singh and Boomsma 2015 give an overarching view on policing across domains, and Riehl and Frederickson 2016 review punishment including policing.

It should be noted that several of these often cited examples for policing and punishment, *e.g.* the ones on legume-rhizobia-mutualism or on yuccas and yucca-moths, should rather be regarded as sanctions (Denison 2000), because they convey an immediate benefit to the actor, *i.e.* the loss of resources (Bergmüller *et al.* 2007, Mills and Côté 2010). On the other hand, Kiers and Denison 2008 "see sanctions as biological analogs of "policing" mechanisms". In the case of mycorrhizal symbiosis, cooperation is also promoted by reciprocal reward (Kiers *et al.* 2011). Punishment however is defined as costly to both actor and recipient (Clutton-Brock and Parker 1995). It is debatable when aggression qualifies as punishment- if all aggressive behaviour is regarded as punishment, because aggression always imposes (usually modest) costs to the aggressor and the opponent, it is ubiquitous. "Real" punishment requires a selfish act of a cheater at the costs of a victim, then the punishment act by the victim (inflicting some costs for the victim, but considerably higher costs for the cheater), and finally some benefit to the

victim at the cost of the cheater (Clutton-Brock and Parker 1995, Monnin and Ratnieks 2001, Raihani *et al.* 2012). The consideration of these costs may be difficult, as also opportunity costs need to be considered. Some of the best examples for "real" punishment are found in the cleaner fish mutualism (Mills and Côté 2010, Raihani *et al.* 2012), most other examples may be examples for coercion, but not for punishment (Raihani *et al.* 2012).

## *1.3 Problems and research questions*

### 1.3.1 Discrimination of worker-laid eggs (WLE)

As reviewed in paragraph 1.2.7, Identification of worker-laid eggs (WLE), it remains elusive how honeybee workers discriminate WLE from QLE. I aimed to identify which chemicals may be used in this discriminatory process. The experimental procedures and results are discussed in chapter 2 (Peptides mark the difference between eggs of queens and workers in honey bees).

### 1.3.2 Specialisation on policing

Division of labour is common in social insects (*e.g.* Wilson 1971, Oster and Wilson 1978) and is likely one reason for their success (but see Dornhaus 2008). In the queenless ant *Gnamptogenys menadensis,* sterile workers (but not gamergates, *i.e.* mated workers) aggress fertile workers (Gobin *et al.* 1999). For *Pachycondyla* ants, it has been suggested that policing is not carried out randomly by all members of a colony, but that some individuals "specialise" in policing (in the sense that they remove more eggs than one would assume based on a Poisson-distribution), and that the policing individuals were not fertile (van Zweden *et al.* 2007). A similar observation has been made in the clonal ant *Platythyrea punctata*, where individuals involved in egg policing were less likely to participate in nest defence (Barth *et al.* 2010) and more likely to become fertile when the current reproductive was removed (Brunner *et al.* 2009a). The same was observed in the ant *Temnothorax unifasciatus,* where individuals who aggressed fertile workers became fertile themselves upon queen removal (Stroeymeyt *et al.* 2007). Also in the Norwegian wasp, *Dolichovespula norwegica*, a specialised subset of workers is performing egg policing (Bonckaert *et al.* 2011a). This confirms the theoretical insight that policing should be carried out by a specialised subset of individuals (Frank 1996).

Honeybees show a remarkable age polyethism, that is many tasks and behaviours are statistically associated with a certain age (Rösch 1925, Rösch 1930, Lindauer 1952, Seeley 1982, Seeley 1983, Kolmes 1985, Kolmes 1986, Seeley 1986, Seeley and Kolmes

1991, Johnson 2008a, Johnson 2010). Additionally to age polyethism, honeybees show also a high degree of task specialisation based on patrilines, *i.e.* the progeny of one of the queen's mates (genetic influences). This has been documented for many traits, including nest defence (Robinson and Page 1988), nest ventilation (Jones *et al.* 2004), undertaking (Robinson and Page 1988, Trumbo *et al.* 1997), water collection (Robinson *et al.* 1984), hygienic behaviour (Rothenbuhler 1964), nectar and pollen collection and others (reviewed in Page *et al.* 2012, Page 2013). It has also been suggested that there are differences in egg removal in queenless honeybee colonies (Robinson *et al.* 1990, Page and Robinson 1994).

Therefore, it seems reasonable to postulate that honeybees may also specialise on policing, and I engaged in testing this hypothesis. This is discussed in more detail in chapter 3, Individual and patriline specialisation in policing behaviour in the European honeybee, *Apis mellifera*.

### 1.3.3 Epigenetics and polyphenism

In honeybees, female larvae can develop into small, sterile workers or large, fertile queens, depending solely on the diet they receive during their larval developmental. Also in other social insects, identical genotypes can give raise to rather different caste phenotypes. Potentially the insects' success in terms of species richness, diversity and biomass is attributable to the widespread occurrence of this polyphenism. Locusts exhibit another stunning polyphenism, namely drastic differences between solitarious individuals that behave similar to common grashoppers, and gregarious individuals that form huge, destructive swarms. Interestingly, some of the physical and behavioural properties can be transmitted to the next generation. Since in the honeybee caste differences are in part epigenetically mediated (Kucharski *et al.* 2008), we had suggested earlier that this was the case in locusts too (Boerjan *et al.* 2011). In chapter 4 (Epigenetics and locust life phase transitions), we review the evidence for an epigenetic regulation of locust phase polyphenism, and propose directions for future research using latest biotechnology. It has been published in slightly different form in The Journal of Experimental Biology (Ernst *et al.* 2015).

### 1.3.4 Theories of and methods to study ageing

Honeybees are also interesting in the light of ageing, because genetically identical individuals can vary considerably in their lifespan (Page Jr and Peng 2001). During spring and summer, honeybee workers often only live for 3-6 weeks (Dukas 2008), whereas in autumn and winter workers live about 6 months (Maurizio 1950, Fluri *et al.* 2012, Fluri

and Gallmann 2013). Queens can even live for several years (Winston 1987, Page Jr and Peng 2001). Some factors and mechanisms governing these huge differences in ageing in bees have been elucidated (*e.g.* Smedal *et al.* 2009). More general theories have been proposed to explain patterns of ageing (for an excellent review see De Haes 2014), and it was from our interest in ageing theories that we reacted to a review article by Selman *et al.* 2012. Selman and colleagues, in a discussion on the status quo of the oxidative stress theory of aging (OSTA), suggest to test this theory in wild populations. We challenge this suggestion in three ways. Firstly, we argue that there is increasing evidence that the basic assumption of OSTA does not hold true. Second, we put forward that animals kept in captivity rather than wild populations are the best choice to investigate the effect of reactive oxygen species (ROS). Finally, we advocate the usage of nonconventional model organisms to reveal whether OSTA is relevant for life history evolution. These questions are discussed in chapter 5 (Life-prolonging measures for a dead theory?) and have been published in AGE (Ernst *et al.* 2014).

Chapter two[21,22]

# 2 Peptides mark the difference between eggs of queens and workers in honey bees

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[21] I designed and performed the experiments and data analysis and wrote the manuscript. My co-authors performed mass spectrometric and statistical analyses, helped with dissections and assisted in writing.

[22] Manuscript in preparation.

## *Abstract*

Honeybees, *Apis mellifera,* are highly eusocial insects with an intricate colony organisation. They show a high degree of cooperation and a reproductive division of labour. There is only one female queen that generally lays all eggs, while the many thousands of female workers remain functionally sterile and altruistically take care of the brood and perform all other tasks in the colony. Occasionally, selfish workers are trying to reproduce and lay eggs as well. However, these eggs rarely ever develop, as they are detected by other workers and cannibalized in a process called "worker policing". These policing workers can accurately distinguish a queen-laid egg from a worker-laid egg, presumably by sensing a chemical signal that the queen provides when laying an egg. This hypothesized queen signal has not yet been identified. Here we show that eggs laid by both queens and workers are coated with a variety of peptides. The peptide quantities differ between worker-laid and queen-laid eggs, with the majority of peptides being more abundant on worker-laid eggs. Several of these peptides are venom constituents, of which the antibiotic peptide melittin was 10.3 times more abundant on worker-laid eggs. In contrast, peptides stemming from transmembrane serine protease (GB54516), were 11.0 times more abundant on queen-laid eggs and were also present in the queen's spermatheca. We suggest that these serine protease derived peptides are the most likely candidate(s) to contribute to the queen signal on eggs.

## 2.1 Introduction

The Western honeybee *Apis mellifera* is a highly eusocial insect with an intricate colony organisation. In other words, these bees form perennial colonies and show cooperative brood care, have overlapping generations and a reproductive division of labour (Michener 1969). A colony is made up by up to 40,000 functionally sterile female workers, several hundred males and only one egg-laying queen. The queen produces up to 2000 eggs per day, whereas the workers usually remain infertile and altruistically perform all other work in the colony, notably foraging and brood care. However, workers have retained the ability to produce viable eggs that will develop into males (Crozier 1975). Exceptionally, some workers will lay unfertilized eggs, but these eggs are usually removed within hours in a process termed "worker policing" (Ratnieks and Visscher 1989). There may be several reasons for this behaviour, the most important ones being low relatedness between workers and male brood sired by other workers on the one hand, and colony efficiency on the other hand (reviewed in Ratnieks *et al.* 2006). Because the queen mates with multiple males (Tarpy *et al.* 2004), workers are, on average, more closely related with offspring of their mother, the queen, than they are with offspring of other workers. Thus, workers increase their fitness by rearing close relatives (queen's sons that are "full" brothers) rather than distant relatives (nephews and "half-nephews"). Therefore selection favours mechanisms to prevent selfish egg-laying or to remove any worker-laid egg (WLE). This requires that policing workers are able to discriminate at high accuracy worker-laid eggs (WLE) from queen-laid eggs (QLE), which is the case in Western honeybees, as well as in other honeybee species, ants and social wasps (Wenseleers and Ratnieks 2006a). Since it is in the interest of both queen and workers that QLE are not removed (Seeley 1985, Keller and Nonacs 1993), it has been hypothesized that queens should mark their eggs with a "queen signal" or "egg marking pheromone" that would label QLE as "do not remove" (Ratnieks 1988). Indeed, in the carpenter ant *Camponotus floridanus*, cuticular hydrocarbons (CHCs) on the egg surface of QLE inform the workers over the presence of the queen and prevent the destruction of QLE (Endler *et al.* 2004), and in the common wasp *Vespula vulgaris*, the CHC 3-methylnonacosane was identified as queen egg marking signal (Oi *et al.* 2015b), which is also (part of) the queen pheromone (Van Oystaeyen *et al.* 2014). So far, the hypothetical queen signal could not yet be identified in the honeybee (Martin *et al.* 2005b, Oldroyd 2015). Several lines of research have been looking into CHCs and organic esters (Katzav-Gozansky *et al.* 1997b, Katzav-Gozansky *et al.* 2001, Katzav-Gozansky *et al.* 2002, Martin *et al.* 2002b, Katzav-Gozansky *et al.* 2003a, Katzav-Gozansky *et al.* 2003b, Martin *et al.* 2004a, Martin *et al.* 2004b, Martin *et al.* 2005a, Martin *et al.* 2005b), but none of these

compounds protected WLE from removal when applied to the eggs. Still, other experiments strongly suggest the presence of a transferable chemical queen signal (Martin *et al.* 2005b). As mostly apolar compounds were investigated in previous research, and because washing QLE in water (M. Beekman, personal communication; this manuscript) or semi-polar solvents like methanol or ethanol (Ratnieks 1995) made these eggs less acceptable for worker bees, we investigated whether more polar compounds such as peptides might be used as a cue or signal on honeybee eggs.

Peptides are used as pheromone signals or cues in taxa as diverse as Bacteria (reviewed in Lazazzera and Grossman 1998), Ciliata (Alimenti *et al.* 2002), Fungi (Duntze *et al.* 1970), and Animalia, including Platyhelminthes (Ghaleb *et al.* 2006), Mollusca (reviewed in Susswein and Nagle 2004), Crustacea (reviewed in Rittschof and Cohen 2004), Insecta (Kubli 1992), Annelida (reviewed in Hardege *et al.* 2004), and Chordata (Singer *et al.* 1986, Kikuyama *et al.* 1995, Chamero *et al.* 2007). Peptides and/or proteins have been found on the body surface of several insects, *e.g.* cockroaches (Korchi *et al.* 1998, Cornette *et al.* 2002, Cornette *et al.* 2003), wasps (Turillazzi *et al.* 2006b, Dapporto *et al.* 2008, Bruschini *et al.* 2011, Baracchi *et al.* 2012), and bees, including honeybees (Zupko *et al.* 1993, Baracchi and Turillazzi 2010). In three investigated species of termites, the body surfaces of queens and kings contain sex-specific compounds of likely proteinaceous nature that correlate with age and reproductive status (Hanus *et al.* 2010). In the termite *Reticulitermes speratus*, the protein lysozyme serves also as egg marking pheromone (Matsuura *et al.* 2007). The paper wasp *Polistes dominula* uses antimicrobial peptides to mark hibernation sites (Turillazzi *et al.* 2006a). The peptides on the abdomen of worker bees and wasps are likely part of the social immune system (Baracchi and Turillazzi 2010, Baracchi *et al.* 2011, Baracchi *et al.* 2012).

It appears thus reasonable to assume that peptides might also play a role in egg discrimination in the honeybee, *Apis mellifera*. In this study, we show that honeybee eggs are coated with differing amounts of peptides on eggs laid by workers and queens, respectively.

## 2.2 Materials and Methods

### 2.2.1 Polar compounds involved in egg recognition

Queens of two honeybee colonies (*Apis mellifera*, predominantly of *carnica* type) were constrained to an artificial comb (Nicot) where they laid eggs in plastic cups mimicking natural worker cells. This allowed to manipulate and transfer eggs without damaging them by touching. To test whether the removal of polar compounds renders QLE less acceptable, eggs were rinsed with a micropipette with a droplet of 7 µL Milli-Q water

(Millipore). In each colony, we introduced in alternating rows artificial cups into the artificial comb (Nicot), using treated (rinsed) (n=22) and untreated eggs (n=44) from both queens. We monitored egg removal after 2 h, 6 h and 24 h. To test whether rinsing with water affects viability which might compromise acceptance by workers, we placed plastic cups with treated (rinsed) (n=110) and untreated eggs (n=132) from four colonies in an incubator at 34 °C and high humidity. We monitored hatching after 24 h, 48 h and 72 h in six replicates.

### 2.2.2 Sample collection procedure for eggs

In the apiary of the KU Leuven, two honeybee colonies (*A.m.*, predominantly of *carnica* type) with natural mated queens (1-3 years old) were used as egg source for queen-laid eggs (QLE). Each colony was split into one queen-right colony and one queen-less colony by moving frames with sealed brood, pollen and honey into new hives. We added young bees into these new colonies by brushing them from brood frames of the queen-right mother colony to ensure the survival of the new colonies. Mother and daughter colonies were regarded as one colony when investigating a colony effect. Freshly laid eggs (< 24 h) were obtained by constricting queens to small areas of empty cells (for QLE), or by adding frames with empty drone cells to the queen-less colonies (for WLE), as workers preferentially lay in the larger drone cells (Gontarski 1938, Free and Williams 1974). 20 eggs were transferred with modified Taber forceps (Taber 1961) into 200 µL cold extraction solution (90 % methanol, 9 % Milli-Q water, 1 % formic acid). The eggs were gently washed by aspiration and blowing out of the extraction solution with a micropipette and the solvent transferred to another vial. The supernatants of 5 samples were pooled (*i.e.* 100 eggs), dried in a vacuum centrifuge and stored at -20 °C until further processing. Samples were resuspended in 25 µL of 2 % acetonitrile, desalted and concentrated using Ziptip® $C_{18}$ (Millipore) following manufactory's instructions, and mass-spectrometric analysed. Three replicates each of two queen-right colonies (QLE) and two corresponding queen-less colonies (WLE) were used *(i.e.* in total 12 samples).

### 2.2.3 Identification of peptides on egg surface

For high resolution accurate mass Orbitrap-analyses (LTQ Orbitrap Velos, Thermo Scientific), 10 µL of the desalted sample was loaded on the trapping column (Pepmap C18, 300 µm x 20 mm, Dionex) with an isocratic flow of 2 % acetonitrile in water with 0.1 % formic acid at a flow rate of 5 µL/min. After 2 minutes, the column-switching valve was switched, placing the pre-column online with the analytical capillary column (Pepmap C18, 3 µm particle size, 75 µm x 150 mm nano column, Dionex). Separation was conducted

using a linear gradient from 2 % acetonitril in water, 0.1 % formic acid to 40 % acetonitril in water, 0.1 % formic acid in 80 minutes. The flow rate was set at 350 nL/min. The mass spectrometer was set up in a data dependent MS/MS mode where a full scan spectrum (350 – 5000 m/z, resolution 60 000) was followed by a maximum of ten CID (Collision induced dissociation) tandem mass spectra (100 to 2000 m/z). Peptide ions were selected as the twenty most intense peaks of the MS1 scan. CID scans were acquired in the LTQ ion trap part of the mass spectrometer. The normalized collision energy used was 35 % in CID. We applied a dynamic exclusion list of 45 sec. Runs were aligned and analysed using Progenesis LC-MS v4.1 (Nonlinear Dynamics) and Peaks Studio v7 (Bioinformatics Solutions Inc.) (Ma *et al.* 2003, Han *et al.* 2011, Zhang *et al.* 2012).

For analyses in Progenesis LC-MS, peptides were identified by means of MS/MS Ion searches using Mascot (v2.2.03) (Perkins *et al.* 1999) against an in-house protein database of the *Apis mellifera* official gene set 3.2 (Munoz-Torres *et al.* 2011, Elsik *et al.* 2014) containing 15,314 sequences (6,447,461 residues). The mass tolerance for precursors and fragments was set to 10 ppm and 0.5 Da, respectively. No enzymatic cleavage was used, but variable modifications (oxidation (M), amidation, and pyro-glutamate formation (N-terminal Q)) were allowed. In Peaks Studio, peptides were identified with the same settings.

### 2.2.4 Identification of peptides in queen's spermatheca

The spermathecae of 6 freely mated, egg-laying queens were dissected in insect saline solution. Peptides were extracted using the same acidified methanol solvent as mentioned above. For this purpose, the spermatheca was submersed in 500 µL cold acidified methanol and subsequently sonicated and centrifuged for 10 min at 16.1 g and 4 °C. The supernatant is transferred and the pellet is resuspended in 200 µL of acidified methanol, sonicated and centrifuged. Supernatants of both extractions are pooled, dried in a vacuum centrifuge and desalted using Ziptip $C_{18}$ (Millipore) as described above. The samples were resuspended (5 % acetonitrile, 0.1 % formic acid) and 5 µL were loaded on a LTQ-device (Finnigan LTQ linear ion trap). Peptides were identified using Peaks Studio v7 with similar settings as above, and the peptide mass tolerance was set to 0.8 Da and the fragment mass tolerance to 1.2 Da.

### 2.2.5 Identification of potential modifications

Raw data files from Orbitrap runs were searched for potential modifications by spectral clustering, using a Bonanza algorithm with the number of matching peaks set to 40 and an E-value threshold of 0.0001 (Falkner *et al.* 2008, Menschaert *et al.* 2009). Based

on the most common shifts in mass, the following potential modifications were expected, accounted for in our data base searches: oxidation and (de)amidation.

### 2.2.6 Identification of signal peptides

Potential signal peptides were identified using SignalP 4.1 using both default settings and sensitive settings (Petersen *et al.* 2011).

### 2.2.7 Gene Ontology

Gene ontology terms were retrieved using standard settings of Blast2Go 3.0 (Conesa *et al.* 2005), and putative gene functions were inferred via NCBI's conserved domain database (CDD) v3.12 (Marchler-Bauer *et al.* 2015).

### 2.2.8 Label-free quantitation of peptide abundances

Relative peptide abundancies were determined using Progenesis LC-MS. Data generated by Progenesis LC-MS were exported and further analysed by an in-house R script (R Development Core Team 2014). An offset of 100 was added to the normalized abundancies to reduce the variation at low intensities. Only peptides that had been identified by both search engines, PEAKS (score > 30) and MASCOT (score > 30) were used (636 features, representing 415 individual peptides). Truncated peptides with overlapping sequences (*i.e.* with additional amino acid residues at the N- or C-terminal) were grouped into one "consensus sequence": when the start position of one peptide falls between the start and end position of another peptide, they are merged into one sequence covering both peptides, and this process is iterated until there are no overlapping peptides left. Overlapping fragments had highly correlated abundancies.

### 2.2.9 Statistics

All statistical analyses were performed in R version 3.2.2 (R Development Core Team 2014). Egg survival in discriminator colonies was analysed with a binomial generalized linear mixed model (GLMM) with time as fixed factor, egg source and discriminator colonies as fixed blocking factors and egg batch (*i.e.* the specific combination of treatment, source and discriminator colony) as a random factor. Egg hatching success in an incubator was analysed with a binomial GLMM with replicate, source colony, age of egg, and treatment as fixed factor. In both cases, the data were weighted by the number of eggs.

Discrimination between caste and colonies were based on principal component analyses (PCA) and logistic discriminant analyses (using both a resubstitution and a cross validation model). Quantitative differences were analysed with linear models for microarray data (limma), p-values were adjusted using the Benjamini-Hochberg-procedure (Benjamini and Hochberg 1995). Heatmaps were generated using the unweighted pair group method with arithmetic mean (UPGMA) and Pearson correlation as distance matrix.

## *2.3 Results*

### 2.3.1 Evidence for polar compounds as signals

QLE that had been rinsed with water (n=44) displayed a greater removal rate than untreated eggs (n=88) (GLMM, type II ANOVA, Likelihood ratio test (LRT) = 7.007, p=0.0081) (Supplementary Table 1). This was not due to their hatchability (as proxy for egg viability) since for treaded eggs (n= 110), it was not significantly different from untreated eggs (n=132) (GLMM, type II ANOVA, LRT = 0.43, p=0.5689) (Supplementary Table 1).

### 2.3.2 Identification of proteinaceous compounds on the egg surface

Using combined searches with Mascot and PEAKS within Peaks Studio, we identified 670 unique peptides (false discovery rate (FDR) < 0.1 %), that could be mapped to 58 proteins. Relying only on peptides that were identified by both search engines, we identified 518 individual peptides that were derived from 36 proteins. Twenty-five peptides are recorded both with and without modification (Supplementary Table 2). The peptide TEMIKDADNSMNS occurred in three forms: twice or single oxidated and not oxidated (M). In total, 82 peptides were posttranslationally modified: 8 peptides were C-terminally amidated, 39 were oxidated (M), 35 were pyroglutamated (Q).

Four peptides matched on two or more proteins: YYSPLASHGLY (GB55211, GB55212), YEDPDTAGNK (GB48505, 52829), RVAPEEHPVL and KSYELPDGQVITIGNE (GB41306, GB41308, GB41310, GB43029, GB44311).

Note that, because of our methodological focus on peptides in the lower mass range (acidic methanol extracts, no enzymatic treatment), each identified peptide should be considered as identification on its own. We identified peptides that are part of larger proteins, but we did not detect the entire protein. However, for the convenience and clarity of the manuscript, we discuss the “protein” identification because multiple peptides map to the same protein.

### 2.3.3 Identification of spermatheca peptides

Mass spectrometric analysis of the queen's spermathecae revealed 20 peptides derived from two proteins, transmembrane serine protease (GB54516) and a protein electronically annotated as chitinase-like protein Idgf4-like isoform X1 (GB52829) (Supplementary Table 3). Five of these peptides were also found on QLE: IGKPISKPIVLR, LETGIGDFFSR, KNPIVRDDFQFVFVNPR, AVNDLGDVLSK, PISKPIVLR. Of the latter, there was also a larger fragment found on eggs. Another four peptides were shorter fragments of peptides found on eggs: PIVRDDFQFVFVNPR, GKPISKPIVLR, PIVRDDFQFV, PIVRDDFQ. No peptide of GB52829 had been detected on eggs.

### 2.3.4 Caste and colony differences in peptide abundancies

We analysed abundancies (relative intensities of peptide fragments; Supplementary Table 4) on three levels: individual identified peptides (Supplementary Table 5, Supplementary Figures 5-8), overlapping peptide sequences ("consensus sequence") (Supplementary Table 6, Supplementary Figures 9-12), and proteins (Supplementary Table 7, Supplementary Figures 13-16). On all levels, we were able to discriminate between QLE and WLE, based on the first principal component (PC1) in a Principal Component Analysis (PCA) (Figure 3, Supplementary Figures 1-2, Supplementary Table 8). The second principal component (PC2) allowed the discrimination between colonies.

On the level of proteins, PC1 separates castes and explains 53.1 % of the variation, and PC2 discriminates between colonies (16.6 % of variance explained) (Figure 3; factor loadings are given in Supplementary Table 8).

The factors contributing the most to the separation of castes (QLE vs. WLE, PC1) were transmembrane serine protease (GB54516), pancreatic triacylglycerol lipase-like (GB43509), LOC100578107 (GB52831), cysteine peptidase LOC100576982 (GB47943), mediator of RNA polymerase II transcription subunit 12-like (GB42182), yellow-g2 (GB55201), and serine protease nudel (GB40567).

The separation between colonies (colony 1 vs. colony 2, PC2) was influenced mainly by cuticular protein 3 (GB48832), general transcriptional corepressor ssn6-like (GB46084), trichohyalin-like (GB50065), hymenoptaecin precursor (GB51223), H9KRW3 (GB54810), and vitellogenin (GB49544).

On the level of consensus sequences, PC1 separates QLE from WLE (caste differences), accounting for 49.8 % of the variance (Supplementary Figure 1, Supplementary Table 8). PC2 distinguishes between colonies and accounts for 20.5 % of the variance.

A similar result was found on the level of individual identified peptides, PC1 (45.5 % explained variance) again separates castes, and PC2 (19.7 %) separates colonies (Supplementary Figure 2, Supplementary Table 8).

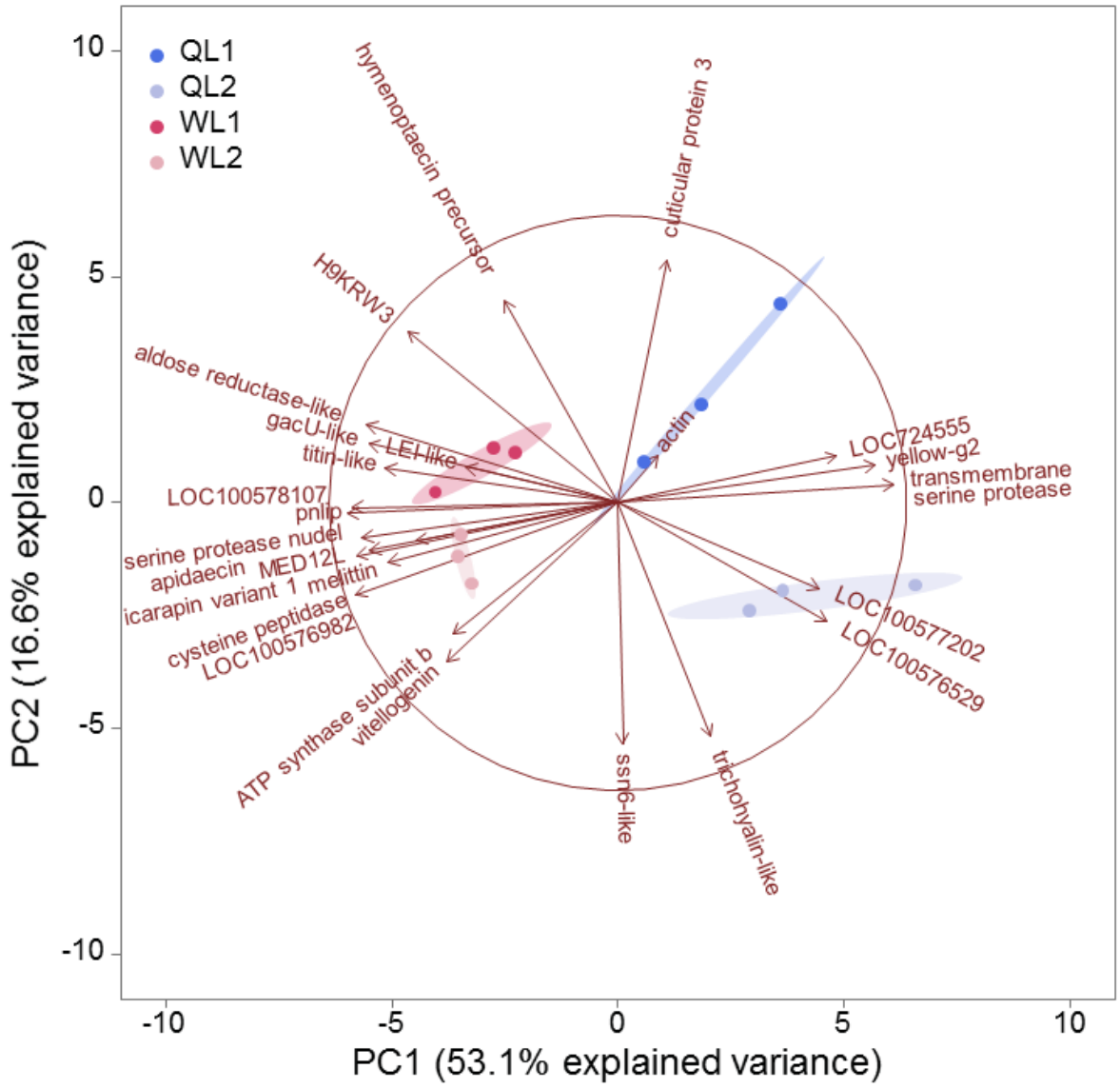


***Figure 3 - Biplot of Principal Component Analysis of proteins.*** *Samples of the same hive cluster together. PC1 explains 53.1 % of the variation and separates worker-laid eggs WLE (red, left side) from queen-laid eggs QLE (blue, right side). PC2 explains 16.6 % of the variation and separates colony 1 (upper part) from colony 2 (lower part). Abbreviations: LEI-like: leukocyte elastase inhibitor-like; MED12L: mediator of RNA polymerase II transcription subunit 12-like; Pnlip: pancreatic triacylglycerol lipase-like; ssn6-like: general transcriptional corepressor ssn6-like.*

In a logistic discriminant analysis, PC1 was the best predictor for caste (likelihood ratio test, $\chi^2$=16.636, p=4.529 $10^{-5}$). A resubstitution model was 100 % correct, whereas a cross-validation model (10-fold cross validation) resulted in 92 % correct classifications (whereby WLE are always correctly identified, and QLE in 83.3 % of the cases). In a logistic discriminant analysis, PC2 was the best predictor for discrimination

between colonies (likelihood ratio test, $\chi^2$=16.6361, p=4.529 $10^{-5}$). A resubstitution model was 100 % correct, whereas a cross-validation model (10-fold cross validation) resulted in 91.7 % correct classifications.

### 2.3.5 Differential occurrence of peptides

On all three levels of analysis, 40-48 % of the investigated features were less abundant on QLE than on WLE, whereas 8-16 % were more abundant on QLE (Figure 4, Supplementary Figures 3-4, Supplementary Table 5-7).

On the level of consensus sequences (Supplementary Figure 3, Supplementary Table 16), 14 out of 92 were more abundant on QLE, especially all six fragments of transmembrane serine protease (GB54516) (between 28.6 and 6.3 times), two fragments of LOC100576529 (GB50066) (3.4 and 2.9 times), and the one fragment of yellow-g2 (GB55201) (2.7 times).

Additionally, several other fragments were more abundant on QLE (trichohyalin-like (GB50065) (4.1 times), mediator of RNA polymerase II transcription subunit 12-like (GB42182) (3.3 times), apidaecin precursor (GB47546) (3.1 times)), while other fragments of the same proteins were more abundant on WLE (trichohyalin-like (GB50065) (3.6 and 2.4 times), apidaecin precursor (GB47546) (3.1 and 2.0 times), and 5 other fragments of mediator of RNA polymerase II transcription subunit 12-like (GB42182) (between 2.6 and 1.8 times)).

A total of 38 consensus sequences were more abundant on WLE, next to the above mentioned especially 7 out of 27 fragments of vitellogenin (GB49544) (up to 18.9 times), the one fragment of melittin precursor (GB44112) (10.3 times), two of pancreatic triacylglycerol lipase-like (GB43509) (6.0 and 5.8 times), one of the hymenoptaecin precursor (GB51223) (5.7 times), icarapin variant 1 precursor (GB40759) (2.5 times), LOC100578107 (GB52831) (3.1 and 2.9 times), all three titin-like (GB52832) (3.4, 3.2, 2.4 times), the one fragment of aldose reductase-like (GB505981) (3.0 times), and three out of four cysteine peptidase LOC100576982 (GB47943) fragments (up to 2.9 times).

Differences between colonies are less pronounced (9 out of 92 consensus sequences differ). In colony 1, one fragment of H9KRW3 (GB54810) was 3.1 times and one fragment of LOC100577202 (GB55095) was 3.9 times more abundant. Yet, 2 others fragments of LOC100577202 (GB55095) were more abundant in colony 2 (8.0 and 1.9 times), as were 2 fragments of vitellogenin (GB49544) (3.4 and 2.6 times), one of trichohyalin-like (GB50065) (5.0 times), mediator of RNA polymerase II transcription subunit 12-like (GB42182) (9.5 times), and general transcriptional corepressor ssn6-like (GB46084) (3.2 times).

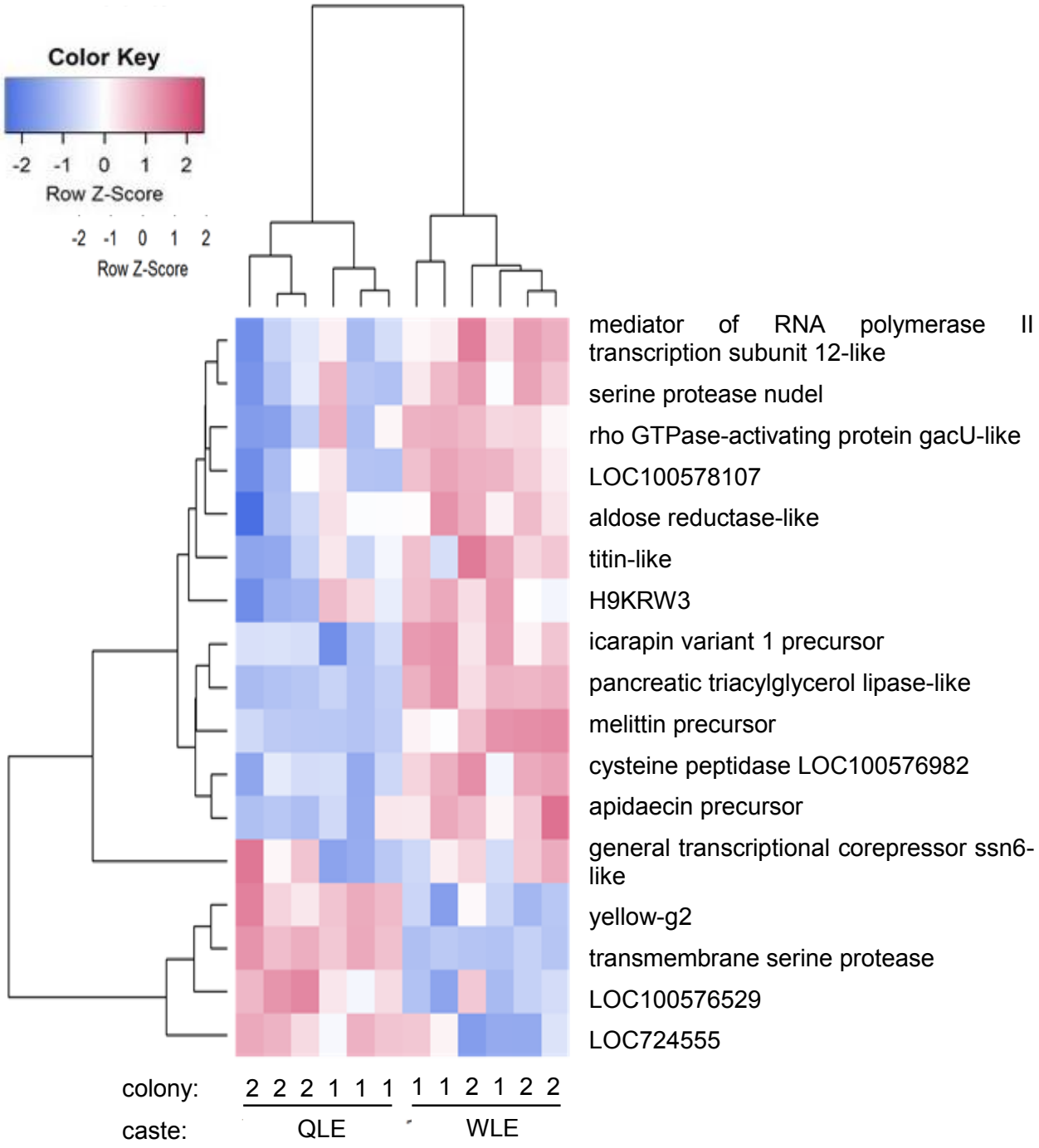


***Figure 4 - Heatmap for abundancies of proteins.*** *Most proteins are less abundant on QLE than on WLE. The samples cluster according to caste (QLE vs. WLE). They also cluster according to colony, with the exception of column 3 and 4 for WLE. Relative abundancies are given as row z-scores.*

On the protein level (Figure 4, Supplementary Table 7), 4 of 25 proteins were more abundant on QLE than on WLE, especially transmembrane serine protease (GB54516) (11 times) and yellow g (GB55201) (2.7 times). On WLE, 12 proteins were more abundant,

notably melittin precursor (GB44112) (10.3 times), pancreatic triacylglycerol lipase-like (GB43509) (5.9 times), titin-like (GB52832) (3.4 times), and aldose reductase-like (GB50598) (3.0 times).

## 2.4 Discussion

### 2.4.1 New candidates for a queen signal

Egg marking pheromones and signalling of fertility have generally been thought to be based on cuticular hydrocarbons (CHCs) (reviewed in Liebig 2010, Oi *et al.* 2015a). For instance, in the common wasp *Vespula vulgaris*, eggs of queens and workers differ in their CHCs composition and resemble the CHCs of their respective mother (Bonckaert *et al.* 2012), and 3-methylnonacosane has been identified as part of the queen signal (Oi *et al.* 2015b). Interestingly in the fire ant *Solenopsis invicta*, venom alkaloids possibly play a role in both fertility signalling and egg marking (Eliyahu *et al.* 2011), and a proteinaceous or protein-associated queen pheromone stored in the venom sac triggers execution of sexual larvae (Klobuchar and Deslippe 2002). It seems possible that the observed prevalence of CHCs in reports dealing with signalling fertility might partly be due to the dominance of organic solvents that are used to extract volatile compounds (cf. Hanus *et al.* 2010). For instance, Hanus *et al.* 2010 found that kings and queens in several termite species are characterised by proteinaceous substances, and Matsuura *et al.* 2010 report that in another termite species a mix of an alcohol and an ester regulate reproductive differentiation. In the ant *Myrmecia gulosa*, CHCs indicate fertility, but CHCs fractions alone were less attractive than a mix of CHCs and more polar extracts, indicating that polar compounds also play a role in recognition (Dietemann *et al.* 2003). For the paper wasp *Polistes dominula*, differences in polar substances on the cuticles between foundresses and workers have been reported (Dapporto *et al.* 2008); these peptides are not colony-specific (Bruschini *et al.* 2011).

Peptidergic/proteinaceous pheromone components have rarely been studied in social insects. For instance, in the paper wasp *Polistes dominula* it could be shown that hibernation sites are marked with antimicrobial peptides (Turillazzi *et al.* 2006a, Turillazzi *et al.* 2006b). To our knowledge our data represent one of the first reports of identified peptides and/or proteins on the surface of eggs of (social) insects (Blum and Hilker 2008). Known examples are the termite *Reticulitermes speratus* that uses the antibiotic lysozyme to mark eggs (Matsuura *et al.* 2007) and the Mediterranean fruit fly *Ceratitis capitata* (Marchini *et al.* 1997). Polar pheromones in insects have also been described in the desert locust *Schistocerca gregaria* (McCaffery and Simpson 1998, Miller *et al.* 2008, Islam 2013), as well as a pheromone binding carrier protein in the Madeira

cockroach *Leucophaea maderae* (Korchi *et al.* 1999). Arguably, also the sex peptide in *Drosophila* and other proteinaceous compounds in seminal fluid of several insects (reviewed in Avila *et al.* 2011) might underline the importance of peptide-based pheromones.

QLE rinsed with polar solvents such as water (experiment 1; M. Beekman, personal communication) and methanol are removed at higher rates than untreated QLE, although the effect was not very strong and did not include a comparison with WLE. We suggest the rinsing procedure does not damage the eggs, given that the rinsed eggs have similar viability as untreated eggs, and that the faster removal of eggs is due to a (albeit incomplete) removal of the queen egg marking pheromone. Thus, it is suggested that the queen signal is transferable and of proteinaceous nature (cf. Ratnieks 1995, Martin *et al.* 2005b). Thompson and colleagues found in a microarray study several venom protein genes up-regulated in anarchistic (fertile) bees and suggested that they "act directly or indirectly as the egg-marking signal" (Thompson *et al.* 2008). Although these components were identified from worker bees, this suggested marker signal was identified from an anarchistic strain of bees in which the WLE are very similar to QLE and escape the removal by other worker bees. Here, we show that the peptide profiles of QLE and WLE differ quantitatively, with some components being up to 228 times more abundant on WLE than on QLE. PCA analyses (Figure 3, Supplemental Figures 1-2) show that this is sufficient to discriminate eggs based on their origin, as expected for a signal compound.

Interestingly, we found that honeybee eggs are coated with antibiotic venom compounds, as was also the case in the termite *Reticulitermes speratus* (Matsuura *et al.* 2007) and the fire ant *Solenopsis invicta* (Vander Meer and Morel 1995). Likely, some of these compounds first served as a defense against microbiological infections before they might have been co-opted as signals (Eliyahu *et al.* 2011), which would be an interesting case of parallel evolution in three taxa. This model of signal evolution would explain the relative ease with which egg marking pheromones seem to have evolved, and at the same time account for the diversity of queen signals.

### 2.4.2 Chemical egg protection

In the social insects, brood items (eggs, larvae, pupae) are protected against robbers and diseases by various behavioural traits (*e.g.* guarding, grooming) and barriers (*e.g.* fortified nests) (Ayasse and Paxton 2008). Additionally, chemicals with antibiotic properties are used to fight viruses, bacteria, fungi, nematodes, and other diseases and parasites (reviewed in Cremer *et al.* 2007). These compounds are collected (*e.g.* plant resins (Chapuisat *et al.* 2007, Simone-Finstrom and Spivak 2012)), produced by symbionts (*e.g.* in the Attine leaf-cutter ants (Currie *et al.* 1999); beewolf *Philanthus*

*triangulum* (Kaltenpoth *et al.* 2005)), or secreted by glands or other body exudates (reviewed in Otti *et al.* 2014). In the honeybee, antimicrobial substances have been found in larval food, honey, and propolis, but also on the comb, as honeybee workers seem to apply venom to it (Baracchi *et al.* 2011). Our finding of venom components and other antimicrobial compounds (see below) on the eggs of both QLE and WLE could support a role for antibiotic peptides in the protection of eggs against brood diseases, as has been found in the fire ant *Solenopsis invicta* (Vander Meer and Morel 1995). Up to now, antimicrobial peptides on the egg surface have been found only once (Blum and Hilker 2008), namely in the med fly *Ceratitis capitata* (Marchini *et al.* 1997). Since the vast majority of eggs in a colony are QLE, we would expect that antimicrobial substances should be more abundant on them than on the rare WLE.

However, venom components are up to 228 times more abundant on WLE than on QLE. Consequently, an alternative but not mutual exclusive hypothesis is that venom compounds rather inadvertently end on the egg surface. Possibly, eggs come in contact with the opening of the poison sac or pass along the sting shaft during the egg laying process, as has been described in ants (Vander Meer and Morel 1995). Differences in the amount of venom compounds found on the egg surface may then be a result in the composition of the venom (the venom of queens containing much less melittin (Inoue *et al.* 1987, Schmidt 1995)), or of the amount of venom that is spread on the egg, depending on the different inner anatomy of queens and workers (see below).

An increased production of venom components might be (indirectly) linked to increased fertility (Thompson *et al.* 2008), as the sting and its associated glands are derived from an ovipositor.

### 2.4.3 Quantitative differences between QLE and WLE and colonies

The peptide profile found on honeybee eggs was caste specific and allows the discrimination between QLE and WLE (Figure 3, Supplementary Figures 1-2). This supports the hypothesis that the queen egg signal is a polar component and likely of proteinaceous nature. In addition, we were also able to discriminate the different source colonies. Indeed, the queen signal is expected to be independent of the colony of origin, but it has been suggested that honeybees are also able to discriminate between eggs of nestmates and non-nestmates (Pirk *et al.* 2007a), which might at least partly rely on peptides. Discrimination of non-nestmate's eggs occurs also in the paper wasp *Polistes fuscatus* (Downing 1991) and the ants *Leptothorax acervorum* (Bourke 1994) and *Formica fusca* (Helanterä et al. 2014, Helanterä and d'Ettorre 2015). How this discrimination occurs has not yet been studied. In the bumblebee *Bombus terrestris*, the chemical profile of CHCs on eggs is both caste- and colony specific (Ayasse *et al.* 1999).

Many peptides are more abundant on WLE than on QLE; potentially, this is due to the ca. five times slower egg-laying process in workers (Martin *et al.* 2004a), which brings the egg in longer contact with compounds on the *bursa copulatrix* (see also 2.4.7, Source of the signal). Compounds more abundant on QLE may stem from the spermathecal gland, which is at most rudimentary in worker bees (Gotoh *et al.* 2013), from other glands associated with the sting and egg-laying (*e.g.* Koschevnikow glands (Lensky *et al.* 1991), sting sheath glands (Martin *et al.* 2005b)), or may reflect caste differences in the composition of the products of these glands.

Unfortunately, our method does not allow us to quantify the absolute amounts of peptides present on the eggs, as different peptides will be ionized with different efficiencies and therefore detected at different rates in the mass spectrometer. It would be desirable to understand which components are most abundant on the eggs, although the quantity does not necessarily predict how easily a compound is detected and recognized.

With our method, we could not identify all detected features, nor are we able to identify polar substances other than peptides. Therefore, we cannot rule out that other polar compounds also play a role in egg recognition. Nevertheless, the differential occurrence of peptides is sufficient to discriminate with high confidence between QLE and WLE, and would therefore not require additional explanation. This is in agreement with observations that > 99 % of WLE are removed, but most QLE remain (Kärcher and Ratnieks 2014).

As our settings for the identification of peptides were stringent (identification by two different algorithms, individual FDR < 1 %, combined FDR < 0.1 %), we may have missed additional peptides; however, we reasoned that false positive results will hinder further research more than some missing compounds.

### 2.4.4 Perception of peptides

Honeybees have excellent chemical senses and are able to not only detect a huge array of floral odours, other highly volatile compounds and $CO_2$, but also less volatile long-chained hydrocarbons (Galizia and Menzel 2001, Brockmann *et al.* 2003, Robertson and Wanner 2006). Their taste perception has been less studied, but seems to be of minor importance when compared to odour perception (Robertson and Wanner 2006, de Brito Sanchez 2011). To the best of our knowledge, it has not yet been investigated if and how honeybees perceive proteins and peptides. As peptides have a rather low vapour pressure, one would expect that they are not easily detected by olfactory senses. However, honeybees are able to smell amino acids (Linander *et al.* 2012), which are not considered to be very volatile either, albeit maybe only at relatively high concentrations. In

vertebrates, MHC peptides perceived by the vomeronasal organ seem to play a role in mate choice, suggesting that peptides could be volatile and sensed (Leinders-Zufall *et al.* 2004). The paper wasp *Polistes dominula* uses venom peptides to mark hibernation sites, and individual wasps show a clear preference for these peptides (Turillazzi *et al.* 2006a, Turillazzi *et al.* 2006b). Therefore, it seems likely that honeybees possess similar sensory abilities.

### 2.4.5 Formation of peptides

Often, we found truncated peptides, *i.e.* peptides with the same core primary structure yet differing in the number of additional N- and/or C-terminal amino acid residues. Because these peptides have been separated by liquid chromatography (different retention times), we can rule out that different truncated forms are an artefact of gas phase fragmentation in the mass spectrometer. The occurrence of truncated peptides has been described earlier for peptides in mouse urine (Sturm *et al.* 2013) (see also section "*source of the signal*"). Often, all peptides mapping to the same protein are regulated in the same direction, *i.e.* all are more abundant on QLE than on WLE, or vice versa. However, the magnitude of the fold changes between QLE and QLE differs considerably between numerous peptides. In several cases, some fragments are more abundant on QLE, whereas other fragments mapping to the same protein are more abundant on WLE (Supplementary Table 8). If peptidases with dissimilar specificities cleave at different rates, *e.g.* because of differences in concentration, specificity, pH-dependency, etc., this may explain why peptides from the same protein are not present in equimolar amounts. This effect could be enhanced if extracellular peptidases also cleave peptides, as they might encounter very different local conditions (between and within queens and laying workers), *e.g.* in the ovaries, oviduct, and *bursa copulatrix*. Indeed, we found a (transmembrane serine) protease (GB54516) that possibly is involved in processing proteins. Additionally, several proteases and peptidases have been identified previously in venom of queens and workers (Chan *et al.* 2013, Li *et al.* 2013a, Resende *et al.* 2013, Matysiak *et al.* 2014, Van Vaerenbergh *et al.* 2014), and as other venom compounds have been detected on the eggs, it is likely that also other cleaving enzymes are present on eggs, and/or that they produce peptide fragments in venom that are then applied to the eggs' surface.

Nineteen of the 25 proteins analysed quantitatively do have a signal peptide and thus likely enter a secretory pathway. Other peptides, however, are fragments from proteins that are not secreted. However, this is not in conflict with a role for communication, as exemplified by mice that use proteolytic fragments for olfactorial recognition of genotypes (Sturm *et al.* 2013).

### 2.4.6 Discussion of the identified compounds

Note that we have not identified complete proteins, but rather peptides mapping on protein sequences. In several cases, only one peptide per protein was found. Here we provide a short overview over the function and occurrence of the 25 proteins that were analysed quantitatively. A more detailed discussion of their potential function and origin is provided in Supplementary Table File 1.

#### 2.4.6.1 Venom compounds and antimicrobial properties

We found peptides stemming from several components of the honeybee venom (icarapin (GB40758), icarapin variant 1 precursor (GB40759), melittin (GB44112), apidaecin (GB47546)). They all were more abundant on WLE. Since the oviduct's trajectory is in close vicinity to the stinging apparatus, it seems likely that some venom leaks from the venom bladder onto the eggs. The lower amounts of melittin on QLE supports this hypothesis, because the queen's venom contains less melittin than worker's venom.

Melittin has also been reported for the spermathecae of virgin and mated queens (Baer *et al.* 2009a); however, melittin is a very sticky peptide that easily binds to all kind of materials and is thus hard to avoid during dissections, and the presence of melittin should therefore be confirmed by qRT-PCR. We cannot exclude that melittin is also present in other glands (potentially) associated with egg laying (Koschevnikow gland, Dufour gland, sting sheath gland).

Several of these venom proteins possess antimicrobial activities, and therefore, the presence of venom components on the eggs' surface might contribute to a defence line protecting the eggs against pathogens (see section on "*chemical egg protection*"). This is supported by the presence of a peptide that belongs to the hymenoptaecin protein (GB51223) that is part of the inducible unspecific immune defence in honeybees (Casteels *et al.* 1993). Here, no differences in abundancy between QLE and WLE have been observed.

If eggs are marked (advertently or inadvertently) by venom components, differences in venom composition between the two castes might be sufficient to create a caste specific chemical profile.

Alternatively, workers might apply more venom onto their eggs than queens do. Because queens lay up to 2,000 eggs per day (Snodgrass 1956), whereas workers only lay at most a few dozen per day (Perepelova 1928 *fide* Ribbands 1953), it might be argued that the venom in queens is spread over many more eggs and is therefore more diluted. However, as the total abundance of peptides on WLE and QLE is negligible in comparison

to the amount of venom both queens and worker contain in their venom bladders, this seems to be not the main reason for the quantitative differences that we report here.

#### 2.4.6.2 Vitellogenin

Several peptides of vitellogenin (Vg) (GB49544) have been identified. Vg is highly abundant inside the eggs (Cardoen *et al.* 2012) and serves as the main energy resource for the developing embryo. In the honeybee, Vg may serve functions other than being a yolk protein, and is involved in the regulation of ageing, foraging, stress resistance and the immune system (Seehuus *et al.* 2006, Nelson *et al.* 2007). Its transcripts are upregulated in the ovaries of laying queens compared to virgin queens (Niu *et al.* 2014).

#### 2.4.6.3 Link to fertility in laying workers

The following eight proteins have previously been linked with worker fertility: mediator of RNA polymerase II transcription subunit 12-like (GB42182), general transcriptional corepressor ssn6-like (GB46084), cysteine peptidase LOC100576982 (GB47943), cuticular protein 3 (GB48832), aldose reductase (GB50598), leukocyte elastase inhibitor-like (GB54541), H9KRW3 (GB54810), rho GTPase-activating protein gacU-like (GB55448). In a whole body transcriptome study, these genes were often highly upregulated in fertile workers compared to sterile workers (Cardoen *et al.* 2011). Our finding of peptides on the egg surface, mapping to these proteins, suggests that the differences reported on the level of the whole body (Cardoen *et al.* 2011) are probably mainly reflecting differences in the ovaries, and that these proteins are enriched on the egg surface.

Leukocyte elastase inhibitor-like (GB54541) did not differ between QLE and WLE. It contains a serpin domain, which often function as serine protease inhibitors (hence the name serpin). If confirmed, this would be of greatest interest, as it might explain differences in the abundancies of peptides (see 2.4.9, Origin of peptide fragments and potential causes for quantitative differences). In short, an inhibitor could influence the activity of proteases (which will produce shorter peptides from larger proteins), and this inhibition will be dependent on the specific environment (pH, salt concentrations, and others) that could be different between queens and workers and thus lead to different activities of the proteases, which could lead to different concentration of peptides.

Potentially, these proteins have functions that are restricted to eusocial insects. For instance, a non-redundant protein blast search of the sequence of GB47943 and GB55448 only delivered hits with sequences of other eusocial hymenopterans (70 % similarity cut-off). Furthermore, considering that all of the peptides from these proteins have been reported to be more abundant on WLE, that they are highly expressed in reproductive

worker bees, and that the expression is mediated by brood pheromone, it could be possible that those proteins are important factors mainly involved in (the regulation of) worker egg-laying.

#### 2.4.6.4 Proteins more abundant on QLE

Comparative analysis, theoretical predictions and experimental evidence all point to the existence of a transferable queen signal. Therefore, compounds that are only present or more abundant on QLE, as the following four proteins were, are of particular interest.

Transmembrane serine protease (GB54516) is the most interesting. On the protein level, it was over 11 times more abundant on QLE than on WLE. This protein is present in the spermathecae of both virgin and mated queens (Baer *et al.* 2009a), and we also found peptides of this protein in queen's spermathecae, suggesting that these peptides are common in queens. This strongly suggests that one or several peptides of GB54516 are (part of) the queen signal on QLE.

Yellow-g2 (GB55201) was 2.7 times more abundant on QLE. Its specific function in honeybees is unknown; however, other members of this gene family are involved in (mating) behaviour, cuticle development, and eggshell formation (reviewed in Li and Christensen 2011), suggesting that it has a function in egg development in the honeybee. The *Yellow-g2* gene was higher expressed in the ovaries of both fertile queens and workers (Niu *et al.* 2014).

Unfortunately, for LOC100576529 (GB50066) and LOC724555 (GB55450), very little information on their function or occurrence is available.

#### 2.4.6.5 Spermatheca-related proteins on QLE and WLE

Next to the above discussed transmembrane serine protease (GB54516), we also identified aldose reductase (GB50598), which occurs in the queen spermathecal fluid and in sperm (Baer *et al.* 2009a), and in semen and seminal vesicle tissue (Collins *et al.* 2006). It was more abundant in ovaries of reproductive workers when compared to inactive ovaries of sterile workers, and had a high transcription rate (top 200 of more than 11,000 probes) (Cardoen *et al.* 2012). This protein has a basic function in energy production. It remains elusive what its function on the egg surface might be. It was more abundant on WLE, indicating that its occurrence is independent of male's sperm (as workers do not mate). Additionally, mellitin (GB44112) and vitellogenin (GB49544) have been found in virgin and mated queen's spermathecae (see above).

#### 2.4.6.6 Proteins in the queen's spermatheca

In the queen's spermatheca, we identified peptides from two proteins, namely transmembrane serine protease (GB54516) (see above), and chitinase-like protein Idgf4-

like GB52829. The function of GB52829 is not clear (see S8). Its transcript levels were higher in queens than in fertile and sterile workers; the two latter did not differ (Grozinger *et al.* 2007). The protein has previously been found in the spermathecae of virgin and mated queens (Baer *et al.* 2009a), as well as in seminal fluid (Baer *et al.* 2009a, Baer *et al.* 2009b). However, we did not detect any peptides of this protein on QLE or WLE.

#### 2.4.6.7 Other proteins more abundant on WLE

Homologues of serine protease nudel (GB40567) are widespread in both social and solitary insects. They are likely involved in the formation of the egg shell.

GB43509 (pancreatic triacylglycerol lipase-like) was 5.9 times more abundant on WLE. BLASTing gave also significant hits for inositol polyphosphate 1-phosphatase-like (GB43508) and pancreatic triacylglycerol lipase-like (GB43510); these three genes lay next to each other on linkage group 11. The exact function of this protein is unknown, gene ontology terms suggest a role in oogenesis, embryo development, and transcription. Surprisingly, this gene has been found to be 5.0 times down-regulated in fertile workers compared to sterile workers (Cardoen *et al.* 2011)[23].

No information is available for LOC100578107 (GB52831), which was 2.5 times more abundant on WLE.

Titin-like (GB52832) was 3.4 times more abundant on WLE. Titin-like proteins are found in insect muscles where they assume partly similar as well as different functions compared to vertebrate titin (reviewed in Bullard *et al.* 2002). Their function in honeybees, and especially in ovaries or eggs, has not yet been studied.

#### 2.4.6.8 *Proteins not differing between QLE and WLE*

These seven proteins did not differ between QLE and WLE: actin (GB41306), ATP synthase subunit b (GB43482), general transcriptional corepressor ssn6-like (GB46084), cuticular protein 3 (GB48832), trichohyalin-like (GB50065), leukocyte elastase inhibitor-like (GB54541), LOC100577202 (GB55095).

Actin (GB41306) is a ubiquitous structural protein; it had been found in seminal vesicle tissue (Collins *et al.* 2006), and another form of actin (GB41310) had been reported for the spermathecae of mated queens (Baer *et al.* 2009a).

ATP synthase subunit b (GB43482) is involved in the ATP production in mitochondria.

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[23] This gene (LOC727190, old bee base number GB11256) has been withdrawn from NCBI, yet seems to be a shorter version of the current GB43509.

GB50065 has been tentatively labelled trichohyalin-like; its function in honeybees is unknown. The same holds true for LOC100577202 (GB55095); homologues were only found in other eusocial insects.

General transcriptional corepressor ssn6-like (GB46084), cuticular protein 3 (GB48832), and leukocyte elastase inhibitor-like (GB54541) have been discussed above in the context of proteins likely involved in fertility.

### 2.4.7 Source of the signal

Chemicals on the surface of eggs may have several origins. They may be components of the egg shell itself, produced and secreted by the egg or the developing embryo inside. They may be added to the surface before or whilst laying, or they may be added or altered after the egg has been deposited, *e.g.* by grooming (*cf*. Lommelen *et al.* 2008). All of these possibilities apply also to the case of honeybee eggs. However, QLE that do not pass the *bursa copulatrix* (*i.e.* dissected out of the ovary) are not protected against oophagy by workers (Martin *et al.* 2004b). Also, since WLE are often removed without any prior contact with workers (pers. observation; Ratnieks 1990b), it is unlikely that the chemical signature of the eggs is altered after laying in a way that would affect egg recognition. Therefore, it is most likely that the queen signal is applied to the egg surface during laying. In the med fly *Ceratitis capitata*, a proteinaceous secretion of the accessory glands is spread onto the egg chorion, most likely during oviposition (Marchini *et al.* 1997). Hover wasps apply a mixture of Dufour gland secretions and nectar on eggs after laying (Keegans *et al.* 1993). In the honeybee, the Dufour gland was suspected to be the source of the egg marking pheromone, although other glands associated with the sting as the Koschevnikow gland could not be ruled out (Ratnieks 1995). Later, this has been disputed, given that the egg does not pass the Dufour gland's opening (Martin *et al.* 2005c). However, our finding of several peptides that usually occur in the venom suggests that gland components may be transferred to the egg. We cannot exclude that several gland secretions are passed on to the cuticle and/or the *bursa copulatrix*, and then transferred during the laying process. Egg-laying in workers is a slow process (ca. 30-45 sec (Page and Erickson 1988) vs. ca. 10 sec in queens (Martin *et al.* 2004a)), which would explain why there are more venom compounds on WLE than on QLE. Also, WLE often stick "to the worker sting area during oviposition" (Martin *et al.* 2004a).

Of greatest interest is the finding of peptides that are much more abundant on QLE and that we have also identified from the spermatheca; a previous study reported the corresponding protein as well in queens' spermatheca (Baer *et al.* 2009a), but not in seminal fluid of drones (Collins *et al.* 2006, Baer *et al.* 2009a, Baer *et al.* 2009b). Likely, compounds of the spermatheca are applied to the egg during laying.

This is most evident for eggs that are intended to develop as female, as the queen can control whether she releases some spermatozoa from the spermatheca to fertilize an egg. As honeybee workers do not discriminate between haploid and diploid eggs (Oldroyd and Ratnieks 2000), we have used diploid QLE. This might have induced a bias, since during fertilization some sperms are released from the spermatheca, and potentially other compounds are transferred to the egg, too. However, in analogy to the various venom compounds, we deem it likely that compounds of the spermatheca are passed onto eggs whether they are fertilized or not. These compounds might also be present in a lower concentration in other secretions, as we have identified them in WLE too (the spermatheca in workers is rudimentary (Gotoh *et al.* 2013)).

### 2.4.8 Link to fertility

Interestingly, some of the peptides that were identified in this study matched to proteins that have been found to be highly upregulated on the transcriptome level in fertile honeybee workers when compared to their sterile sisters (Cardoen *et al.* 2011). These proteins and their derived peptides might be physiologically closely linked with fertility processes, e.g. ovary development, egg formation and fertility-related gland secretion. This is corroborated by findings of Thompson *et al.* 2008 who report that anarchistic reproductive worker bees had higher transcription rates of vitellogenin (GB49544) and also of some venom proteins.

### 2.4.9 Origin of peptide fragments and potential causes for quantitative differences

Nineteen out of the 25 identified proteins contain a signal peptide and are thus likely secreted. Others are not necessarily expected to be found on the egg surface, *e.g.* ATP synthase subunit b (GB43482). Yet, similar proteins have also been found in the mucus and testis of drones, the spermatheca of queens (Chan *et al.* 2013), and in worker venom (Chan *et al.* 2013, Li *et al.* 2013a). In venom, additional traces of non-secretory proteins have been detected (Baer *et al.* 2009a, Baer *et al.* 2009b, Li *et al.* 2013a, Resende *et al.* 2013, Van Vaerenbergh *et al.* 2014), suggesting that some intracellular components are naturally occurring in glandular secretions. Protein and peptide fragments are likely the result of cleaving enzymes (peptidases, endoproteases).

When peptidases cleave proteins at different rates, this would result in differing abundancies of peptides even when the protein abundancies have been the same originally (different protein or peptide quantities applied to the egg being the trivial explanation for quantitative differences). Differences in cleaving rates are not only dependent on the abundancy of the proteases, but also on activation and inhibition,

respectively. In a proteomic study, the cleaving enzyme dipeptidyl peptidase 3-like (GB44541) was found in the ovaries of workers, and was 1.7 times more abundant in fertile workers (Cardoen *et al.* 2012), strongly suggesting that cleavage of proteins and peptides takes place in the ovary. Queen venom contains several peptidases and a trypsin-like protein (Chan *et al.* 2013), and also worker venom contains a form of trypsin (Chan *et al.* 2013) and other peptidases, indicating that cleavage occurs also in the venom reservoir. Because venom components have been found on eggs, this could likely explain the occurrence of different peptides. In this study, we found two proteases (transmembrane serine protease (GB54516) and serine protease nudel (GB40567)) and one potential inhibitor of serine proteases (leukocyte elastase inhibitor-like (GB54541)) that could contribute to the observed caste differences between QLE and WLE.

### 2.4.10 Peptides as artefacts of the isolation method?

The presence of protein fragments lacking a signal peptide might be interpreted as leakage of the eggs. However, if there was leakage of eggs, one would expect to find many more peptides, especially the most abundant ones. Conversely, if extracts of damaged eggs would contain additional peptides, this would be a strong hint that the eggs were not damaged. Our matrix assisted laser desorption ionization (MALDI) mass spectrometric analyses of extracts of damaged eggs show additional peaks when compared to extracts of presumable intact eggs (data not shown), confirming that egg damage did not or only to a small extent occur.

### 2.4.11 Future perspectives

While the data presented here strongly support a role of peptides in the recognition of WLE (peptides differ in abundance on QLE and WLE (Figure 4, Supplementary Figures 3-4, Supplementary Tables 5-7), and washing with polar solvents makes QLE unacceptable), this has not yet been confirmed experimentally. Ideally, it should be tested in a bioassay whether WLE are protected from being eaten when artificial peptides found to be more abundant on QLW are applied to WLE. Additionally, the source of the signal should also be elucidated, and analyses of Dufour glands of queens and workers are currently undertaken.

Several research groups have reported the presence of additional modifications of peptides, *e.g.* phosphorylation of melittin and other venom peptides (Li *et al.* 2013a, Resende *et al.* 2013). Additionally, several isoforms of melittin are known to occur (Sciani *et al.* 2010, Resende *et al.* 2013, Park *et al.* 2014). However, these variants seem to occur

at relative low frequencies, and therefore we did not take them into account for the present study.

### 2.4.12 Conclusion

We identified several peptides on the egg surface of WLE and QLE. Several of these peptides have been found previously in honeybee venom (icarapin, icarapin variant 1 precursor, apidaecin, melittin), which, together with hymenoptaecin, all have antimicrobial functions. This suggests that honeybee eggs are coated with protective components against pathogens. Additionally, we report peptides mapping to proteins that have been implied in fertility regulation previously. Finally, we identified peptides from a protein previously found in queen's spermatheca that are more than ten times more abundant on QLE, and likely candidates to be the queen signal on QLE. We confirmed the presence of the peptides of this protein in queen spermatheca. This protein is likely to be restricted only to eusocial hymenopterans, which also suggests a specific role in the foundation of eusocial insect societies.

## *2.5 Acknowledgments*

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Chapter three[24,25]

# 3 Individual and patriline specialisation in policing behaviour in the European honeybee, *Apis mellifera*

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[24] I co-designed and performed the experiments, did the data analysis and drafted the manuscript. My co-authors co-designed the experiments, performed statistical analyses and assisted in writing.

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## *Abstract*

Worker policing by egg-eating, *i.e.* selective removal of worker-laid eggs, is common in the eusocial Hymenoptera and is regarded as a prime example of kin selection theory. Specialization in policing behaviour has been shown in ants and wasps, but not yet in honeybees. We individually labelled all workers of two colonies and studied their behaviour in a policing assay inside an observation hive. Next, we genotyped workers by microsatellite analysis to determine patriline contributions. We found strong evidence for individual specialisation. Additionally, we show for the first time patriline specialisation in policing, suggesting a heritable component to policing behaviour. Age, however, did not predict specialisation, and workers of all ages except those younger than 5 days and older than 40 days engage in policing.

## 3.1. Introduction

### 3.1.1 Division of labour and specialisation

A high degree of division of labour is thought to be the main reason for the ecological success of eusocial insect colonies (Wilson 1971, Oster and Wilson 1978, Hölldobler and Wilson 1990, Robinson 1992, Hölldobler and Wilson 2009). Usually, they exhibit a reproductive division of labour, where one or few individuals reproduce and most colony members perform all other tasks. Non-reproductive individuals often show further specialisation, generally based on morphological or age grounds (physical castes and temporal polyethism, respectively) (Johnson 2008a, Johnson 2010). In the Western honeybee *Apis mellifera* L., the single queen lays virtually all of the eggs, and the several thousand workers specialise in various sets of tasks, the most obvious difference being foraging (older bees) and in-hive tasks (younger bees) (Butler 1609, Rösch 1925, Rösch 1927, Rösch 1930, Lindauer 1952, Sakagami 1953, Sekiguchi and Sakagami 1966, Seeley 1982, Seeley and Kolmes 1991, Johnson 2008a, Johnson 2008b, Johnson 2010). Further specialisation, partially based on patriline, can be observed, *e.g.* between pollen and nectar foraging (Hellmich *et al.* 1985), or specialisation for particular tasks like undertaking or guarding (Robinson and Page 1988, Trumbo *et al.* 1997). Mechanistically, specialisation can be explained by the response-threshold model (reviewed in Beshers and Fewell 2001). In short, this model proposes that a given task is performed until the threshold to perform another task is reached, which leads to a switch in tasks (Bonabeau *et al.* 1996). The thresholds vary with physiological state, which is, among others, dependent on individual experience and genetic background (Smith et al. 2008b, Jeanson and Weidenmüller 2014). Therefore, individuals experience differing thresholds for different tasks, resulting in specialisation (reduced switching of tasks).

Classically, specialisation is assumed to increase (colony) productivity through improved individual efficiency and reduced costs of switching between tasks (Smith 1776, Duarte *et al.* 2011, Goldsby *et al.* 2012). There is clear evidence for improved efficiency in castes differing physically, *e.g.* reproductive queens vs. workers, and major workers vs. minor workers in some ants (Dornhaus 2008 and references therein). For physically not differing workers however, there is little evidence that specialists are better at the tasks they specialise in (Dornhaus 2008), maybe apart in the case of tasks that involve complex learning, suggesting that it is mainly reduced switching costs that select for specialisation (Goldsby *et al.* 2012, Jeanson and Lachaud 2015).

### 3.1.2 Conflict, conflict resolution and policing

Although the honeybee queen is highly specialized in egg laying and therefore presumably most efficient therein, workers have retained the ability to lay eggs (Bourke 1988). This is advantageous when the queen is lost and attempts of replacing her have failed, in which case ca. 10-40 % workers will develop their ovaries and start laying unfertilized eggs (workers cannot mate) to rear a last batch of males before the colony dies out (Miller and Ratnieks 2001). However, this also bears the potential for conflict over male paternity, *i.e.* whether the queen or the workers should lay male eggs, and which of the workers (Ratnieks *et al.* 2006). Indeed, even in queenright colonies, some workers (0.01-0.1 %) produce viable eggs (Ratnieks 1993, Visscher 1996). Most likely, this is because they are more closely related to their own offspring than with any other male offspring. Yet, the vast majority (> 98 %) of these eggs (ca. 7-9 % of all unfertilized eggs in a colony (Visscher 1996)) usually are efficiently detected and removed (Ratnieks and Visscher 1989), and workers with developed ovaries are attacked (Sakagami 1954, Velthuis 1976, Schneider and McNally 1991, van der Blom 1991, Visscher and Dukas 1995, Dampney *et al.* 2002, Malka *et al.* 2008). Both egg eating and aggression are forms of worker policing, by which workers suppress each other's reproduction in favour of the queen (Ratnieks 1988). This efficient policing lead to the suppression of the conflict and is likely also the cause for the worker's self-constraint (self-policing, acquiescence), *i.e.* most workers do not try to reproduce (Wenseleers *et al.* 2004a, Ratnieks *et al.* 2006, Wenseleers and Ratnieks 2006b, Ratnieks and Wenseleers 2008).

### 3.1.3 Specialisation in policing

Earlier, it had been suggested that in the ponerine ant *Pachycondyla inversa*, workers are specialized in aggressive policing towards workers with developed ovaries, and in policing by egg eating (van Zweden *et al.* 2007). Barth and colleagues (2010) report specialization in aggressive policing for a clonal ant, *Platythyrea punctata*. Finally, Bonckaert *et al*. 2011a describe specialisation in policing by egg-eating in the Norwegian wasp, *Dolichovespula norwegica*. However, policing behaviour in the European honeybee *Apis mellifera* has not been investigated in this respect. This is surprising, given that the honeybee is the best-studied insect concerning division of labour, being the classic model to study worker policing (Bourke 2011, Zanette *et al.* 2012).

In this study, we investigated whether individual workers do specialise in worker policing by egg eating. We also studied whether specialisation on egg-eating is correlated with age. Finally, this is the first study to link specialisation in policing to patrilines.

## 3.2 Material & Methods

### 3.2.1 Observation hive and marking bees

Experiments were performed at the bee keeping facility of the University of Ghent (Belgium) in 2009 and replicated at the bee keeping facility of the KU Leuven (Belgium) in 2010. In each year, a colony of *A. m. carnica* with a naturally mated queen was housed in a three-frame observation hive inside a building. The observation hive was connected to the outsides via a plastic tube to allow bees to forage freely. The queen was not restricted in any way, but brood was removed regularly before bees emerged. Daily, we marked 100 newly emerged bees individually with Opalithplättchen (Graze, Weinheim, Germany, and Ewa Podlewska, Fabianki, Poland) and acrylic paint on their abdomen (Amsterdam All Acrylics, standard series, Royal Talens, Apeldoorn, The Netherlands, and deco craft, Lefranc & Bourgeois, Le Mans, France). These bees were offspring of an unrelated, naturally mated queen. Following standard procedures, a sealed brood comb was placed at an incubator at 34 °C and high relative humidity, and daily all emerged bees were removed. Marked bees were kept overnight on top of the observation hive, separated by a wire mesh from the inside, to allow bees to get adjusted to the colony odour (Seeley 1995), and were allowed to enter the colony the next day. By following this procedure for several weeks before and throughout the experiment, virtually all bees inside the observation hive were individually labelled.

### 3.2.2 Observation of policing behaviour

To observe policing behaviour, we introduced worker-laid eggs (WLE), using several unrelated queenless colonies as egg source, and queen-laid eggs (QLE) of several unrelated queenright colonies. Using modified Taber forceps (Taber 1961), eggs were transferred in alternating rows of WLE (n=33-69) and QLE (n=33-69) into an experimental comb (ca. 9.5 x 7 $cm^2$), built by bees on a glass plate. The experimental comb was introduced into the observation hive in close vicinity to brood, to allow bees of all ages easy access to the eggs. Bee behaviour was recorded for two hours with a high definition camera (Panasonic HDC-HS 300K). Experiments were repeated on several days, occasionally two experiments were conducted on the same day.

### 3.2.3 Patriline analysis

At the end of the experiment, the colony in the observation hive was anesthetized with $CO_2$, placed on ice and bees were individually stored at -80 °C until further analysis. DNA was extracted following a modified Chelex-method (Walsh *et al.* 1991) by crushing a hind

leg with a pestle in liquid nitrogen, adding 200 µL of a hot Chelex 100 suspension (10 %, Biorad) and subsequent incubation for 15 min at 95 °C. Samples were centrifuged for 10 min at 13000 rcf and 50 µL supernatant was stored at -20 °C. Two touch-down multiplex-PCR reactions were carried out in 10 µL volumes, containing 5 µL master mix (Qiagen multiplex PCR kit), 3 µL milliQ water, 1 µL primer mix and 1 µL crude DNA-extract. Primers were fluorescently labeled with VIC, PET, NED and FAM (Applied Biosystems). The first multiplex reaction contained primers Am005, Am43, Am56, and Am107, the second Am46, Am59, Am98, and Am125 Solignac *et al.* 2003, each at 0.2 µM final concentration. PCR conditions for the first multiplex reaction were as follows: initial denaturation at 95 °C for 15 min; ten cycles of 30 sec at 94 °C, 90 sec at 60 °C, and 90 sec at 72 °C, whereby the annealing temperature was reduced by 0.2 °C at each cycle; 25 cycles of 30 sec at 94 °C, 90 sec at 58 °C, 90 sec at 72 °C; a final extension of 10 min at 72 °C. The second multiplex reaction was similar, except that the annealing temperature decreased from 62 °C to 60 °C in the first ten cycles, and was set to 60 °C for the next 25 cycles. PCR products of the two multiplex reactions were run together on an ABI-3130 Avant capillary sequencer by mixing 1 µL of each PCR reaction, 7.8 µL formamide and 0.2 µL Genescan 500 LIZ size standard (Applied Biosystems) and analysed with GeneMapper software (Applied Biosystems). Patrilines were assigned manually. We estimated the non-sampling error (NSE, *i.e.* the likelihood to miss a patriline because it was not sampled) and the non-detection error (NDE, *i.e.* the chance to miss a patriline because it is indistinguishable, with a given set of genetic markers, from another patriline) following Boomsma and Ratnieks 1996. More specifically, $NSE = (1-p)^n$ with n = sample size, and p = proportion of workers sired by the undetected male, and $NDE = \prod_j^k \sum_i^k (q_i)^2$ with $q_i$ = the proportion of the $i^{th}$ allele of the $j^{th}$ genetic marker (*e.g.* allozyme, here: microsatellite). Following Starr 1984, we estimated effective paternity $M_e = {}^1/_{\sum_i^k p_i^2}$ with $p_i$ as the $i^{th}$ paternity (proportion of workers sired by the $i^{th}$ male), because our large sample size did not require the estimators suggested by others (Pamilo 1993, Nielsen *et al.* 2003).

### 3.2.4 Statistical analysis

Two-sided Fisher's exact tests (α= 0.05) were used to test for differences in survival of queen-laid eggs (QLE) and worker-laid eggs (WLE), as well as for differences in age between policing and non-policing workers.

A generalized linear mixed model (GLMM) fitted by maximum likelihood with gamma distribution, individual as random factor and scaled age and reaction to cell content as fixed effect was used to test for differences in the time a bee spends in a cell, depending

on whether the cell contained an egg or not, and whether the egg was removed or not. This was followed by Tukey post-hoc tests.

To test for specialisation on policing based on individual and patriline, we applied a binomial generalized linear model (GLM) with the following fixed factors: colony, patriline nested in colony, and individual nested in patriline, using expected contributions (based on genotyping results) as custom intercept. The expected contribution per patriline equals the proportion of workers of this patriline in the colony. We calculated the expected proportion of eggs removed by patriline as the product of the proportion of the patriline and the sum of removed eggs.

Because three patrilines did not police any eggs, we did not include them in our binomial model due to separation issues. Two-sided Fisher's exact tests ($\alpha$= 0.05) have been used to investigate patriline specialisation including these three extreme cases. Observed number of removed eggs were compared to theoretical expected values as described above.

All tests were performed in R 3.1.2 (R Development Core Team 2014).

## *3.3 Results*

### 3.3.1 Behavioural observations

As expected, bees removed both WLE and QLE, yet QLE survived longer than WLE, confirming that bees discriminated against WLE. In ten experiments in 2009, 77 QLE and 15 WLE survived 12-24 h, which differs significantly from an equal distribution (46 QLE vs 46 WLE) (Fisher's exact test, $p=1.82 \times 10^{-6}$). In 2010, out of 414 eggs removed during the two hours observation periods, 60 % (250/414) were WLE and 40 % (164/414) were QLE, which differs from an equal distribution (207 QLE vs. 207 WLE) (Fisher's exact test, p=0.0033). Also on the individual level, most bees removed both WLE and QLE. However, as reported earlier Ratnieks 1990b, eggs were often not removed on the first visit to the cell.

Bees that removed eggs usually entered a cell with their head and thorax. A bee will not always immediately back out of the cell when an egg is removed, but may stay in the cell for another 20 sec. On three occasions, two workers removed eggs with their head hardly inserted into the cell. Likely, the egg is reached with a bee's tongue, and then transported to the mouth.

Bees that ate an egg sometimes cleaned their tongue and/or antennae afterwards, more often they move directly into an adjacent cell. Occasionally, bees rotate inside a cell (ca. 180°, rarely 360° or more), yet this has been observed with and without egg removal. Most of the time, bees kept their wings folded back; in rare instances, bees entered cells

with their wings halfway spread open. Occasionally, their hind legs did not grasp the comb but rather hung loosely when entering a cell. On one occasion, a worker was licking the ground of the cell around the egg, occasionally touching the egg with her forehead, but without removing the egg. Another worker was working on the cell wall of a cell before finally removing an egg.

### 3.3.2 Duration of egg eating

Visits to cells (Figure 5) were shorter on average when the cell was empty (t=6.7 sec, sd=6.45 sec, n=47) than when no egg was removed (t=9.7 sec, sd=14.8 sec, n=31), which in turn was shorter than when an egg was removed (t=28.8 sec, sd=28.7 sec, n=227) (GLMM, $\chi^2$=48.2582, df=2, p=3.318x10$^{-11}$; Post hoc Tukey tests with adjusted p-values: empty vs. not removed, z=3.014, p=0.00658; not removed vs. removed, z=-3.764, p< 0.001 ; empty vs. removed, z=-5.902, P< 0.001). We did not find an effect of age (GLMM, t=1.214, p=0.225) or individual. The median age was 17 days, average 18.2 days (sd = 4.9 days).

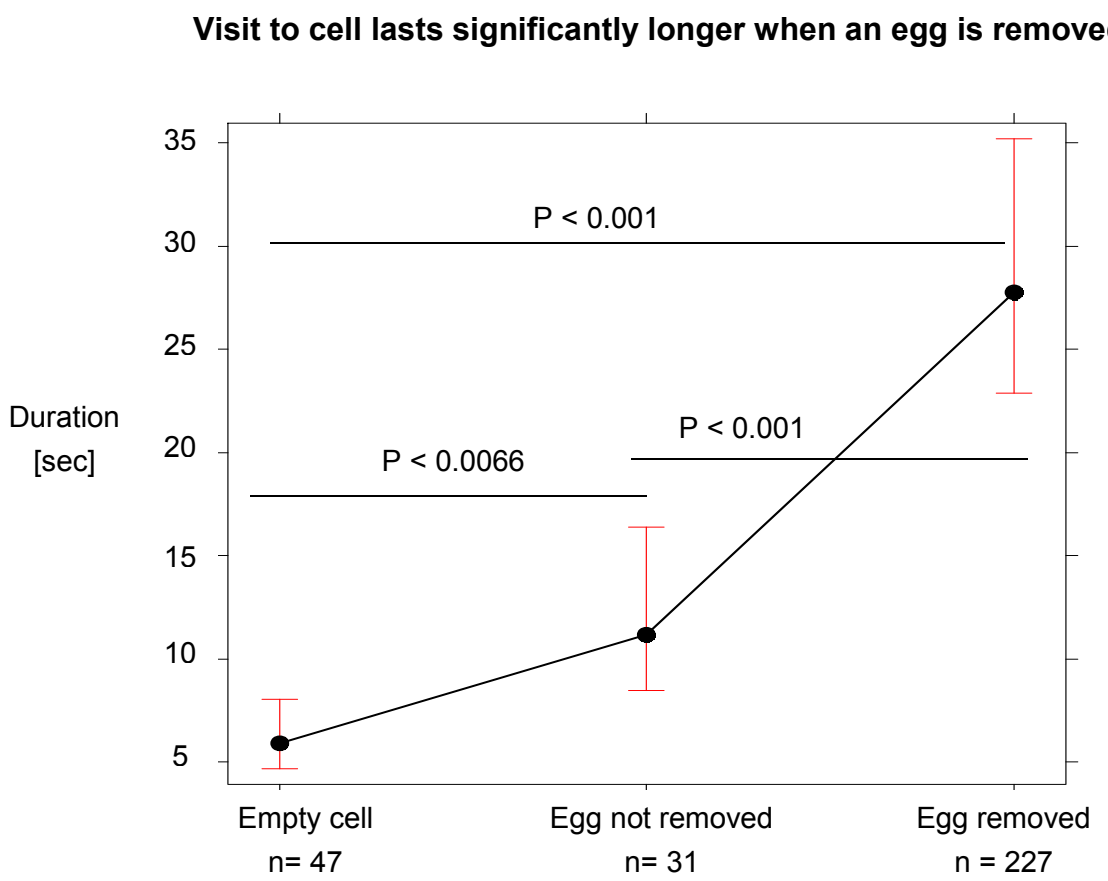


***Figure 5 - The average duration of visits to cells is shorter when a cell is empty or when an egg is not removed than when an egg is removed.*** *Whiskers show lower and upper 95 % confidence intervals. All Tukey post hoc tests p < 0.01 (empty cell vs. egg not removed, empty cell vs. egg removed, egg not removed vs. egg removes).*

However, egg removal can be very fast (within 2 sec on several occasions by different workers), and one worker removed 3 eggs in only 24 sec, spending 2, 3, and 7 sec in the cells. Conversely, bees sometimes also remain in cells after they had removed an egg. Thus, the variance within and between workers is high.

### 3.3.3 Patrilines

We detected 8 patrilines ($M_e$ = 5.88) in 2009 and 7 patrilines ($M_e$ = 4.17) in 2010. The non-sampling error (NSE) for a patriline contributing 10 %, 5 %, or 1 % to the worker population in our colonies was $5.71 \times 10^{-10}$, $3.16 \times 10^{-5}$, and 0.13, respectively in 2009 (n=202), and $7.16 \times 10^{-9}$, $1.08 \times 10^{-4}$, and 0.17, respectively, in 2010 (n=178). The non-detection error (NDE) was 0.0097 in 2009 and 0.0015 in 2010.

### 3.3.4 Age of policing bees

Based on bees that we had identified during the instantaneous sampling regime, we did not find differences in age between bees that had removed eggs and those that have not been observed eating eggs (data for 2009 in Figure 6) (Fisher's exact test, 2009: p=0.116, 2010: p=0.3098). In 2009, bees that removed eggs were on average 22.9 days old (sd= 7.3 days, n= 34), whereas control bees were on average 25.2 days old (sd= 7.7, n=184). In 2010, policing bees were on average 25.1 days (sd= 9.2 days, n= 45) vs. control bees 26.0 days (sd= 8.3 days, n= 759).

### 3.3.5 Specialisation

Our second GLMM found strong evidence for individual specialisation (likelihood ratio test, $X^2$=26.361, $p=2.832 \times 10^{-7}$). Our third GLMM indicated patriline specialisation (likelihood ratio test, $X^2$=44.325, $p=2.781 \times 10^{-11}$). Our binomial GLM with colony, patriline nested in colony, and individual nested in patriline as fixed factors, using expected contributions (based on genotyping results) as custom intercept, did not find significant differences between the two colonies (p=0.788), but differences between individuals ($p=1.81 \times 10^{-6}$), and differences between patrilines (corrected for the expected contribution per patriline based on the frequency in the colony, $p=6.59 \times 10^{-8}$) (Figure 7). We could not detect any effects of age on policing behaviour. When we included the patrilines that did not remove any eggs, patriline differences were found for both years (Fisher exact test, 2009: p=0.0007755, 2010: $p= 1.758 \times 10^{-12}$) (Figure 7).

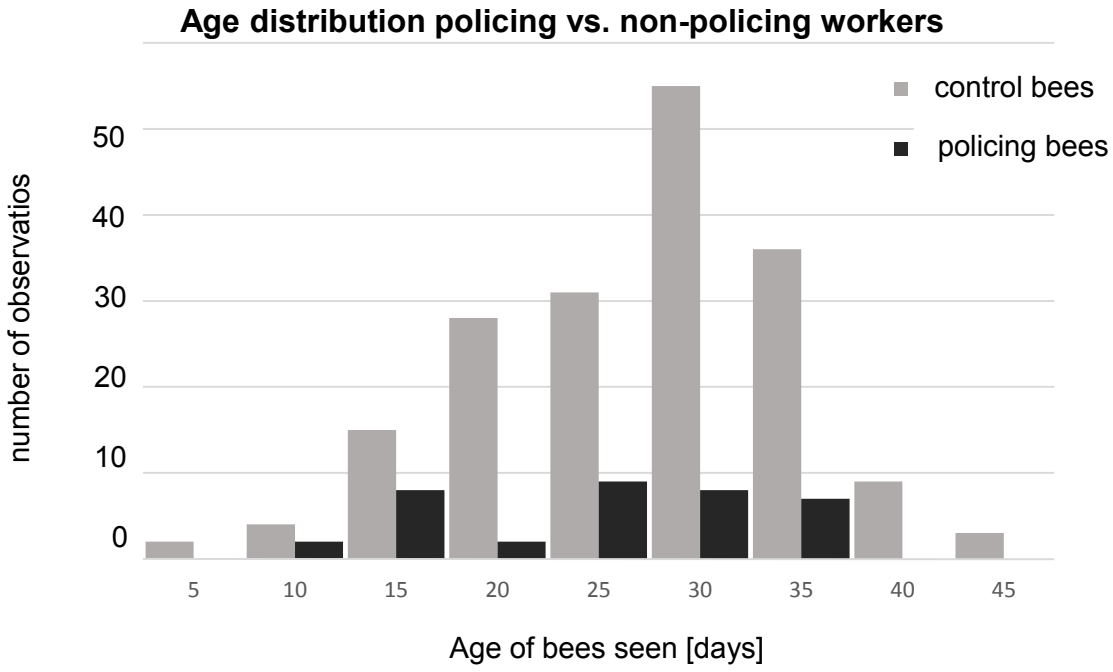


***Figure 6 - There was no difference in age between bees that removed eggs vs. bees that did not.***

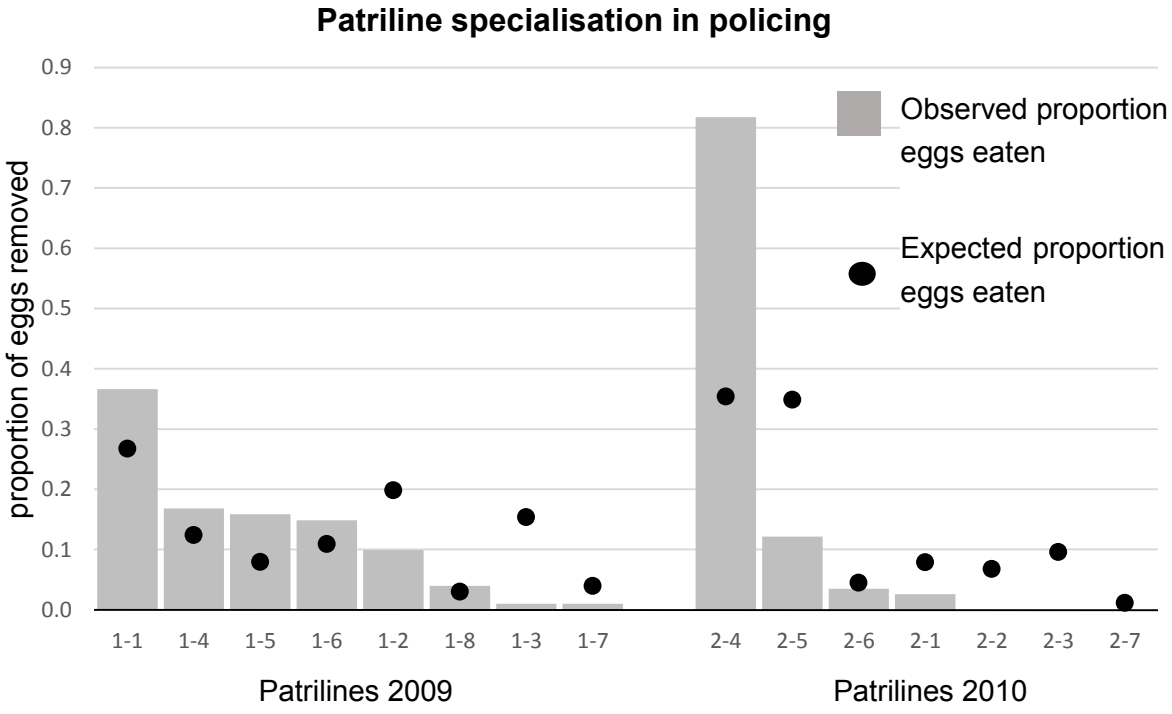


***Figure 7 - Observed vs. expected proportions of eggs removed per patriline****. Some patrilines remove more eggs than expected based on the number of their members, others remove less than expected.*

## 3.4 Discussion

### 3.4.1 Behaviour

Honeybee workers often inspect one cell after another, occasionally omitting one or two. Sometimes, they inspect one cell for 20-30 sec, leave the cell, and re-enter it, even when they had just removed an egg inside this cell.

The general survival of eggs after 12-24 h was lower than in other reports (Ratnieks and Visscher 1989, Martin *et al.* 2005b), suggesting that eggs have been damaged during the transfer, and/or that the conditions for rearing brood in the experimental comb were less than optimal. However, bees seemed to accept the experimental comb, as they occasionally deposited food in the cells, and on one occasion an egg hatched into a larva. Bees removed both WLE and QLE, but QLE survived longer, indicating that eggs were not removed undiscriminatingly. For this study, it was irrelevant whether policing occurs on hygienic (*e.g.* Velthuis *et al.* 2002, Gadagkar 2004, Pirk *et al.* 2004, Nonacs 2006), relatedness (*e.g.* Ratnieks 1988, Ratnieks and Visscher 1989, Beekman and Oldroyd 2005), or other grounds. For future studies, it would be interesting to investigate whether bees that are involved in hygienic behaviour (*e.g.*, undertaking, removal of diseased, damaged, or dead brood) are also more likely to engage in worker policing by egg eating. Similar, it would be of interest whether policing workers are more likely to activate their ovaries, either under queen-right and/or queen-less conditions. The former could be interpreted as selfish (corrupt) policing (if a worker would replace an egg with her own egg) (Wenseleers *et al.* 2005), the latter as reproductive competition (Velthuis *et al.* 2002).

In *Dolichovespula* wasps, egg eating is often accompanied by rotating in the cell or up- and downward movements (Wenseleers *et al.* 2005, Bonckaert *et al.* 2011a). In contrast, while bees sometimes rotated in a cell, this was not consistently associated with egg eating, and no other characteristic movement or behaviour had been observed that would allow an easy detection of egg removal behaviour.

It has been suggested that policing evolved in defence against social parasitism (Pirk *et al.* 2007b, Tan *et al.* 2009); potentially, the disturbance of the colony during the insertion of the experimental comb (containing WLE and QLE) might have triggered workers to reject more eggs, as this could be a situation where social parasites might have entered the nest. We observed a high degree of agitation during the insertion (including stinging behaviour), which calmed down within minutes after the insertion was completed.

As expected, bees spent the least time in a cell when it was empty. They remained only marginally longer in a cell when they encountered an egg that they did not remove. This suggests that they required some time to decide whether to remove the egg, or that the

presence of an egg somehow impaired their activity in the cell. When bees decided to remove an egg, they spent on average considerably more time in a cell. This might be due to a more careful inspection of the egg, or a prolonged phase of cleaning after they removed the egg. Bees do not always clean a cell after egg removal, as witnessed by removal times as short as 2 sec. Conversely, some bees spent several minutes inside a cell. Likely, they were working wax, as some have been observed to gnaw on cell rims, too.

### 3.4.2 No age specialisation

The age distribution of the non-policing bees that were seen on the experimental frame at the instant sampling points follows roughly a normal distribution (Figure 6), where only few bees are older than 40 days, as expected for short-lived summer bees (Winston 1987). Relatively few bees younger than 10 days have been observed. This is surprising at first sight given that we introduced every day 100 1-day-old bees. However, young bees are found mostly in the centre of the brood nest where they are engaged in cleaning cells and brood care (Seeley 1982, Kolmes 1985, Kolmes 1986, Seeley 1986, Seeley and Kolmes 1991, Johnson 2008a). Since the experimental comb was introduced above the brood nest and due to the construction separated by wooden frames from other comb, this could have prevented more young bees from entering the experimental comb. Policing bees differed not significantly in age from non-policing bees, although they seemed to be more evenly distributed over the different age classes. This might be due to a relatively low number of identified policing bees. Based on the pronounced age polyethism in honeybees, we would not have been surprised to find evidence for age specialisation. On the other hand, bees of all ages are inspecting cells ("patrolling") (Lindauer 1952, Johnson 2008a, Johnson 2008b) and thus encounter eggs. From that point of view, there would be no advantage of specialisation in policing, unless bees' discriminatory skills vary with age. Indeed, older foraging bees have better appetitive associative learning skills than young hive bees, and very young (< 1 week) and very old bees learn relatively poorly (Ray and Ferneyhough 1997, Morgan *et al.* 1998, Ray and Ferneyhough 1999, Behrends and Scheiner 2009, Behrends and Scheiner 2012). On the other hand, young (5-8 days) and middle-aged hive bees can form long-lasting memories (Arenas and Farina 2008).

To date it remains unknown whether these differing learning abilities have an influence on the ability of discriminating QLE and WLE.

Several unmarked or unidentifiable bees have removed some eggs. These may have been drifted bees from other colonies, old bees still present in the colony during the experiments (and thus older than 50 days), or bees that have lost their marks. Because

the chance that the (paint) marks are lost increases with age, our estimate of the age might be slightly biased to younger ages.

### 3.4.3 Patriline specialisation

The queen mating frequencies are in the same range as reported earlier, albeit lower than the average of 14 (Tarpy and Nielsen 2002, Tarpy *et al.* 2004, Tarpy *et al.* 2010, Tarpy *et al.* 2015), indicating that our observation of patriline specialization is a natural phenomenon and not due to an unusual number of matings. The NSE is low, indicating that we have not missed a significant number of patrilines due to under-sampling. We calculated a NDE, based on the colony data rather than on the population (as this data was not available), to estimate a potential underestimation of rare patrilines. Our results suggest that the genetic markers used were highly variable and sufficient to detect any patriline well below the NSE. Thus, if we missed patrilines, they likely contributed less than five percent of the worker population, and therefore did not influence our conclusions.

For many behaviours, specialisation based on patrilines has been reported (Robinson 1992). This is expected in a response-threshold model, where different (genetically underpinned) thresholds determine the likelihood of performing a given task (Beshers and Fewell 2001, Lattorff and Moritz 2013). However, thresholds are not only influenced by genes, but also by ontogeny (development), physiology (hormones), experience, and other factors modulating individual motivation (Beshers and Fewell 2001, Duarte *et al.* 2011, Jeanson and Weidenmüller 2014). For instance, learning and self-reinforcement can lead to specialisation. Thus, in cases where the genetic predisposition is of minor importance, we expect to find specialisation not being linked to patrilines, whereas in the other cases it is likely to be found. Our finding of specialisation on policing based on patrilines suggests that ontogeny and learning are less important in this trait, or that genetic determinants of learning are involved.

### 3.4.4 Individual specialisation

Individual experience can increase the chance of performing a task (*e.g.* Ravary *et al.* 2007, Chittka and Muller 2009, Van Wilgenburg *et al.* 2010). Thus, bees that have removed an egg might be more likely to remove another egg. This does not imply that bees that remove eggs are better at discriminating between QLE and WLE, although it seems plausible that learning through increased experience eventually leads to better and/or faster discrimination. In this study, we introduced a relatively large amount of WLE (30-81) at a high concentration (in less than 170 cells). It has been estimated that in

a large bee colony (of 30-40,000 bees), ca. 15-40 WLE per day are deposited Visscher 1996; in our observation hives, ca. 3,000 bees were present, suggesting that only $\frac{1}{10}$ (i.*e.* 1-3 WLE) would naturally occur. The chances to encounter a WLE are thus usually slim in a queenright colony, with only 4-14 % of male eggs laid by workers (Visscher 1996), especially in smaller colonies where workers are usually closer to the queen and thus less likely to activate their ovaries (Orlova and Hefetz 2014). Therefore, while the effect of policing is spectacular (selective removal of WLE), actual egg eating is rather rare, and policing bees would spend most of their time inspecting many cells before encountering a cell containing a WLE. Potentially, the increased exposure to WLE triggered more bees to police and to remove eggs, or increased the removal rate per policing bee, *e.g.* by lowering their acceptance threshold, as has been observed for nest guards in response to an increase of intruders (Couvillon *et al.* 2008).

### 3.4.5 Limitations of the study

The data presented here have been obtained from only two colonies of Carniolan bees (*A. m. carnica)*. As policing behaviour may vary between subspecies (Kärcher and Ratnieks 2014), colonies (Ratnieks 1995, Martin *et al.* 2005b), seasons (Ratnieks 1993, Visscher 1996) and between individuals (this study), the results of this study cannot be extrapolated to general worker policing behaviour. Having said that, the specialisation of patrilines and the individual specialisation appear to be robust. Some bees could not be identified, and because older bees are more likely to lose their marks, this might have biased our data to younger ages.

### 3.4.6 Future perspectives

Bonckaert *et al.* 2011a and Wenseleers *et al.* 2005 have observed selfish policing in wasps, *i.e.* a worker removes an egg to replace it with an egg of her own. It would be interesting to test whether the same holds true for honeybees, *i.e.* whether policing workers have developed ovaries. Detailed behavioural observations will be required to reveal which other tasks are performed by policing bees, especially whether they are involved in hygienic behaviour such as undertaking, or whether they are foraging. Additionally, brain proteomics and transcriptomics could reveal more differences that explain why some workers do remove eggs, while others do not.

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Chapter four[26,27]

# 4 Epigenetics and locust life phase transitions

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locust phase; polyphenism; locust swarming; *Locusta migratoria*; *Schistocerca gregaria*; *Apis mellifera*; invertebrate; DNA methylation; histone modification; methylome

[26] I helped in coordinating the writing, contributed to writing, and made Figure 10. Boris Van Hiel and Geert Depuydt wrote the vast majority of this manuscript and designed tables and supplemental materials. Fabian Ernst photoshopped Figure 8 that made it to the cover.

[27] This article has been published as Ernst *et al.* 2015 in the Journal of Experimental Biology, where it was also used as cover image. It has been slightly modified for this thesis.

## *Abstract*

Insects are one of most successful classes on earth, reflected in an enormous species richness and diversity. Arguably, this success is partly due to the high degree to which polyphenism, where one genotype gives rise to more than one phenotype, is exploited by many of its species. In social insects for instance, larval diet influences the development into distinct castes; and locust polyphenism has tricked researchers for years into believing that the drastically different solitarious and gregarious phases might be different species. Solitarious locusts behave much as common grasshoppers. However, they are notorious for forming vast, devastating swarms upon crowding. These gregarious animals are shorter lived, less fecund, and transmit their phase characteristics to their offspring. The behavioural gregarisation occurs within hours, yet the full display of gregarious characters takes several generations, as does the reversal to the solitarious phase. Hormones, neuropeptides, and neurotransmitters influence some of the phase traits, however, none of the suggested mechanisms can account for all observed differences, notably imprinting effects on longevity and fecundity. This is why more recently, epigenetics has caught the interest of the polyphenism field. Accumulating evidence points towards a role for epigenetic regulation in locust phase polyphenism. This is corroborated in the economically important locust species *Locusta migratoria* and *Schistocerca gregaria*. Here, we review the key elements involved in phase transition in locusts and possible epigenetic regulation. We discuss the relative role of DNA methylation, histone modification, and small RNA molecules, and suggest future research directions.

## 4.1 Introduction

The term epigenetics tends to take a variety of meanings (Jablonka and Lamb 2002, Haig 2004). In its narrow sense, it can be defined as "meiotically and mitotically heritable changes in gene expression, not based on DNA sequence alterations" (Riggs *et al.* 1996). In a broader sense, it can also be interpreted as "modifications of chromosome structure" (Bird 2007). Independent of the interpretation, however, epigenetics did not receive much attention in insect research until recent years.

The most prominent epigenetic mechanisms, namely (1) methylation of cytosine in DNA, (2) modifications of histone proteins, (3) nucleosome positioning and regulation by non-coding RNA, were only rarely the focus of the entomology field. This may have been due to the general low, often almost undetectable, levels of methylation in insects and other invertebrates (Glastad *et al.* 2011), including the prime model organisms *Drosophila melanogaster* MEIGEN 1830 and *Caenorhabditis elegans* (MAUPAS 1900), suggesting that DNA methylation in insects is of minor importance (Glastad et al., 2011). However, it should be noted that the detection of methylated bases in DNA and/or RNA is prone to errors, especially when the methylation rate is relatively low. As a result, methylation levels, or even its occurrence *per se*, have been heavily debated in *C. elegans* (Klass *et al.* 1983, Simpson *et al.* 1986) and *D. melanogaster* (Achwal *et al.* 1983, Lyko *et al.* 2000, Raddatz *et al.* 2013).

The discovery of a functional DNA methylation system in the European honeybee *Apis mellifera* LINNAEUS 1758 (Wang *et al.* 2006) triggered a renewed interest in the role of epigenetics in insect biology. In addition, recent advances in sequencing technologies have tremendously facilitated the systematic study of epigenomes and epigenetics in a wide range of insects (Krauss *et al.* 2009, Lyko *et al.* 2010, Walsh *et al.* 2010, Zemach *et al.* 2010, Bonasio *et al.* 2012, Lo *et al.* 2012, Smith *et al.* 2012, Zwier *et al.* 2012, Weiner *et al.* 2013, Xiang *et al.* 2013, Ye *et al.* 2013, Beeler *et al.* 2014).

The diversity of epigenetic systems in insects has made them interesting models to understand DNA methylation (Lyko and Maleszka 2011). The observation that locust DNA may be the subject of slightly higher methylation levels (1.3 % – 1.9 % of total cytosines, depending on the tissue) than reported for other insect species Boerjan *et al.* 2011, revived the interest in epigenetic control in locust phase polyphenism. Polyphenism, where one genotype produces several phenotypes, is relatively common in the Animal Kingdom (Simpson *et al.* 2011). Popular examples in vertebrates include temperature-dependent sex determination in some fish and reptiles, where the ambient temperature experienced during a specific time in development triggers the development into male or female (Navarro-Martin *et al.* 2011). The insect clade provides some of the very best models for polyphenism (reviewed in Whitman and Ananthakrishnan 2009,

Moczek 2010, Simpson *et al.* 2011). Metamorphosis in holometabolic insects, where larva, pupa, and imago often differ dramatically in various traits, is just one example. Other well-studied examples include the formation of eye-spots in the butterfly *Bicyclus anynana* (BUTLER 1879) (reviewed in Brakefield and Frankino 2009), or the striking difference in horn size in dung beetles (*Onthophagus*) (Kijimoto *et al.* 2013). In aphids it has been shown that both wing polyphenism – *i.e.* the development of winged or wingless morphs – and morph polyphenism – *i.e.* oviparity or viviparity – are maternally regulated (Zera and Denno 1997, Hartfelder and Emlen 2012, Ogawa and Miura 2014). In addition, research into polyphenism and developmental plasticity has often relied on the caste phenomenon in social insects.

Locusts undergo similar drastic changes when they start swarming, a behaviour that has been extensively studied for many years (Pener and Simpson 2009, Burrows *et al.* 2011, Wang and Kang 2014). When solitary locusts become gregarious, they form enormous groups of countless individuals, spanning occasionally hundreds of square kilometres (Ferenz 1990). The socio-economic impact of these swarms is estimated to be up to several billion US$ (www.fao.org/ docrep/018/i2940e/i2940e17.pdf). In this review, we will focus on the epigenetic aspects of polyphenetic transitions in the most important and best studied species, the migratory locust *Locusta migratoria* (LINNAEUS 1758) and the desert locust *Schistocerca gregaria* FORSSKÅL 1775.

## 4.2 Comparing solitarious and gregarious phases: morphology, behaviour and physiology

Phase transition between the solitarious and the gregarious form encompasses extreme phenotypic plasticity at multiple levels including locust morphology, behaviour, neurochemistry, and physiology (Uvarov 1966, Pener and Yerushalmi 1998, Pener and Simpson 2009, Verlinden *et al.* 2009) (Figure 8, Table 1). Among the most obvious effects of phase transition are changes in morphological appearance including body size and colour (Uvarov 1966, Pener and Simpson 2009). Solitarious individuals are generally bigger and cryptically coloured compared to their long-term gregarious counterparts that have a conspicuous bright body colour (Figure 9). More subtle anatomical differences can be seen in the shape and size of eyes, wings, antennae, and jumping hind legs, as well as in the distribution of sensory receptors (Pener 1991, Pener and Yerushalmi 1998). In addition, phase transition induces a broad range of physiological differences in lifespan, metabolism, immune responses, endocrinology, and reproductive physiology (Pener and Yerushalmi 1998, Verlinden *et al.* 2009, Wang and Kang 2014). Higher fecundity and smaller eggs have been observed in solitarious versus gregarious forms of desert and migratory locusts (Maeno and Tanaka 2008, Maeno and Tanaka 2009). Increased

population density also alters the rate of sexual maturation, but effects are species-dependent: gregarious desert locusts sexually mature more rapidly, whereas the opposite has been reported for *L. migratoria (*Norris 1950, Norris 1952, Maeno and Tanaka 2009). In gregarious male desert locusts, this is accompanied by a bright yellow colouration due to the incorporation of yellow protein into the cuticle (Wybrandt and Andersen 2001, Sas *et al.* 2007).

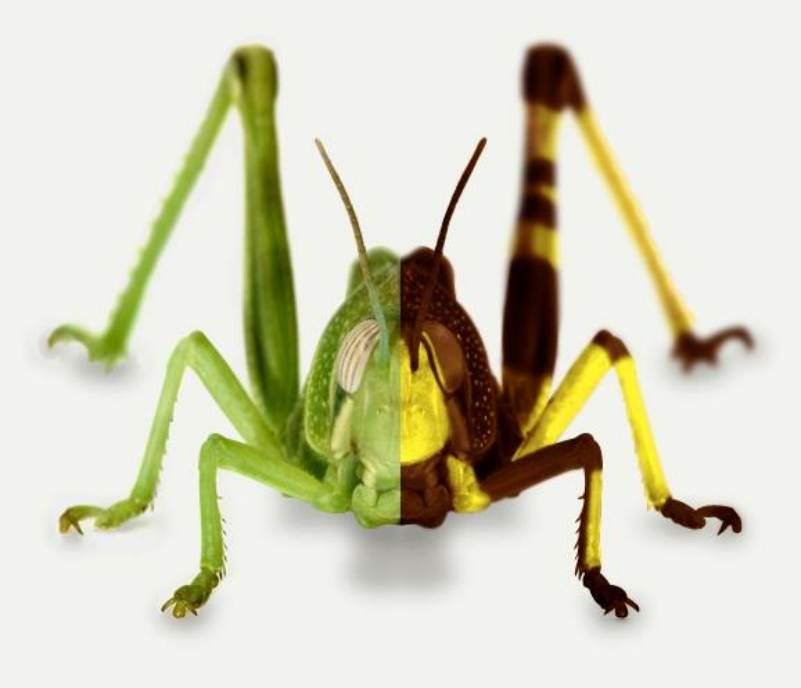

***Figure 8 - Photomontage of a solitarious (left) and gregarious (right) Schistocerca gregaria.*** *Crowding induces gregarisation and is perceived via (1) repeatedly touching of the hind legs, and/or (2) the sight of locusts. Gregarisation results in altered colour, morphology, physiology, and behaviour. Pictures courtesy of Tom Fayle.*

Behavioural dissimilarities are the most intriguing phase-related differences. Solitarious locusts normally avoid each other, but increased population density can rapidly trigger attraction to other locusts resulting in aggregation behaviour that can lead to the generation of devastating migratory swarms (Uvarov 1966, Rogers *et al.* 2003, Pener and Simpson 2009). Gregarious morphs exhibit a wider dietary range, display increased locomotory activity, and will fly predominantly during day-time, in contrast to isolated locusts that generally fly at night (Uvarov 1977, Pener 1991).

Related to behavioural changes imposed by group living and foraging, phase transition in locusts has been found to alter brain structure and functioning. Long-term gregarious desert locusts have a smaller body size, but their brain is substantially larger – about 30 % – than that of long-term solitarious locusts (Ott and Rogers 2010). In addition, Ott and Rogers reported that the relative distribution of brain regions differs

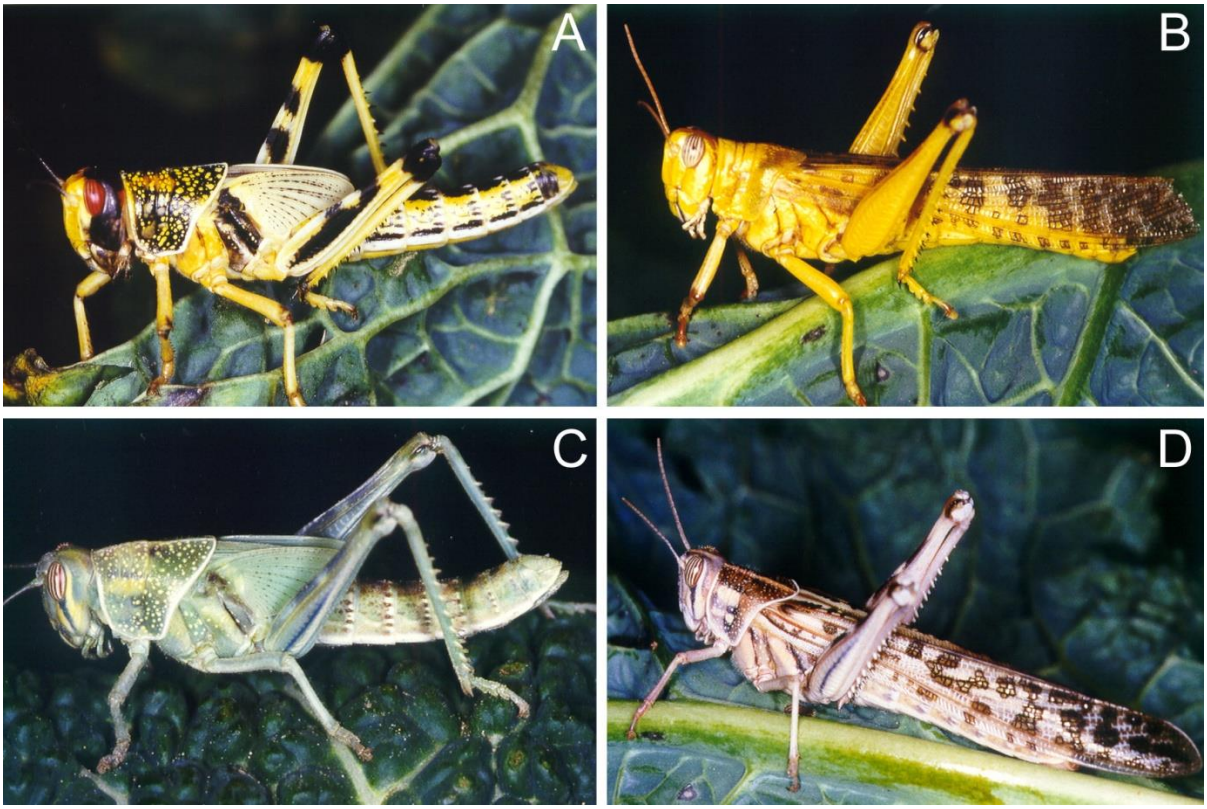


***Figure 9 - Body colouration of Schistocerca gregaria depends largely on phase and developmental stage.*** *Solitarious locusts are larger and cryptically coloured, gregarious locusts display aposematic colours. Males are shown in the gregarious phase as last instar larva (A) and imago (B), and in the solitarious phase as last instar larva (C) and imago (D).*

between the two phases. Solitarious locusts invest more in lower-level sensory processing, reflected by their relatively large primary olfactory and visual neuropils. In contrast, the larger brains of gregarious locusts are more dedicated to the integration of sensory cues in higher-processing regions, which is thought to support their lifestyle as generalist forager in dense, migratory swarms where competition among group members is high (Ott and Rogers 2010). Other changes in brain functioning situate at the level of circuit activity and function (Fuchs *et al.* 2003, Ayali *et al.* 2004, Blackburn *et al.* 2010, Burrows *et al.* 2011). For example, gregarious *L. migratoria* show reduced habituation of interneuron activity in response to approaching objects. Phase transition also affects associative learning in the desert locust (Simoes *et al.* 2013). Long-term solitarious locusts learn more quickly to associate an odour with an aversive food source, an effect that can be overridden by crowding, enabling locusts to adopt a different feeding strategy. Furthermore, brain functioning is affected by changes in the neurochemistry of the locust's nervous system upon phenotypic transformation (Rogers *et al.* 2004, Anstey *et al.* 2009, Verlinden *et al.* 2009).

Solitarious locusts can switch to gregarious behaviour in only a few hours (Ellis 1953, Ellis 1962), whereas other changes in colour, morphology, and reproductive physiology alter on a much slower timescale (Roessingh *et al.* 1993, Pener and Yerushalmi 1998, Simpson *et al.* 1999, Pener and Simpson 2009). Reversible transition between both phases occurs gradually and through intermediate forms, spanning multiple generations. Rapid behavioural changes can rely on short-term neuronal plasticity to alter circuit activity and function, whereas morphological responses such as in brain structure or muscle morphology depend on long-term remodelling that can span several generations (Tanaka and Maeno 2006, Simpson and Miller 2007, Burrows *et al.* 2011). This suggests that epigenetic signals accumulate when reinforced, or fade away with time when they are not reinstalled, which would account for the slow change of some phase characters (Jablonka and Raz 2009, Burggren 2015) (Figure 10).

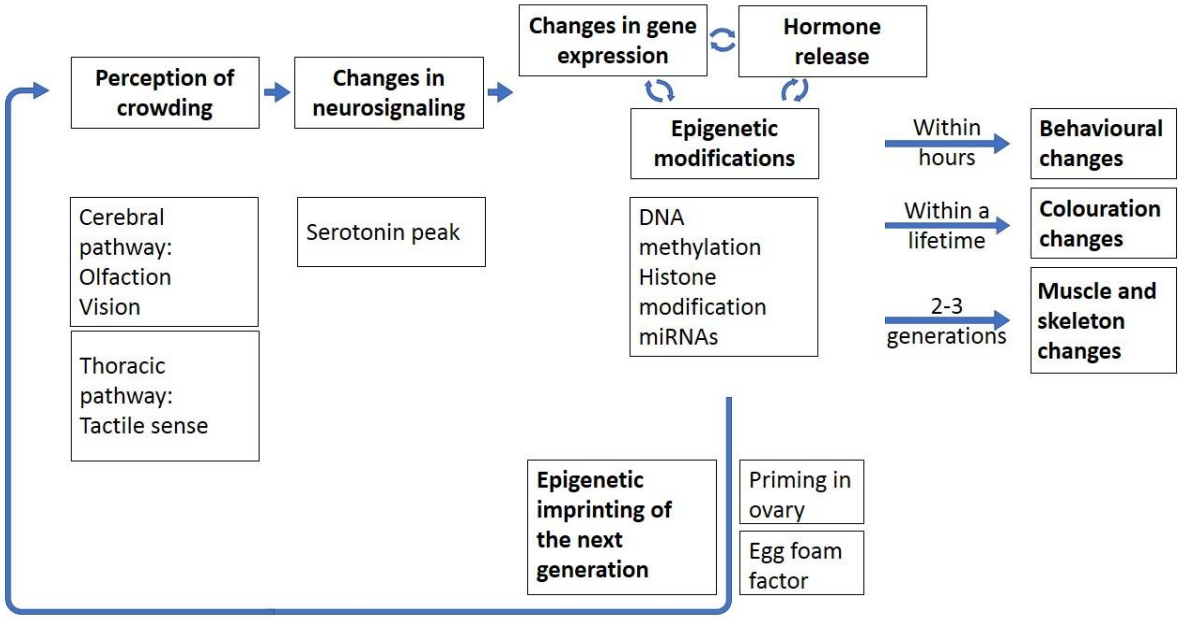


***Figure 10 - Hypothetical model for epigenetic remodelling in locust phase transitions.*** *Crowding causes profound differences in neuronal and hormonal signalling, gene expression, and epigenetic modifications, that eventually lead to significant changes in behaviour, physiology, and morphology on different timescales. Hormones, gene expression, and epigenetic modifications influence each other. Epigenetic marks will perpetuate these changes over moultings and egg formation. Eggs may be primed in the ovary and in the egg pod by an egg foam factor. When offspring also experience crowding, epigenetic alterations may accumulate that subsequently lead to morphological changes and the phenotype of long-term gregarious locusts.*

***Table 1: Examples for differences in morphology, physiology, behaviour, and molecular changes between solitarious and gregarious locusts of Schistocerca gregaria FORSSKÅL 1775 and Locusta migratoria (LINNAEUS 1758).***

| *Schistocerca gregaria* | | *Locusta migratoria* | |
|---|---|---|---|
| Solitarious | Gregarious | Solitarious | Gregarious |
| **Morphological differences** | | | |
| Large females, small males | Smaller females, large males | Large females, small males | Smaller females, large males |
| Long wings | Short wings | Long wings | Short wings |
| Convex pronotum | More concave pronotum | Convex pronotum | More concave pronotum |
| High F/C ratio[1] | Low F/C ratio | High F/C ratio | Low F/C ratio |
| Low E/F ratio[2] | High E/F ratio | Low E/F ratio | High E/F ratio |
| More sensilla on antennae | Less sensilla on antennae | More sensilla on antennae | Less sensilla on antennae |
| More ovarioles | Less ovarioles | More ovarioles | Less ovarioles |
| More, smaller eggs | Fewer, bigger eggs | More, smaller eggs | Fewer, bigger eggs |
| Light hatchlings | Dark hatchlings | Light hatchlings | Dark hatchlings |
| Beige adults | Yellow adults | Green adults | Darkbrown females/ yellow males |
| Green/beige nymphs | Black and yellow nymphs | Green/lightbrown nymphs | Black and orange nymphs |
| 5-6 nymphal stages | 5 nymphal stages | 5-7 nymphal stages | 5 nymphal stages |
| Long hind legs | Shorter hind legs | | |
| 5-6 eye stripes | 5 eye stripes | | |

| **Morphological differences (continued)** | | | |
|---|---|---|---|
| Large eyes | Small eyes | | |
| Small brain | Larger brain (30 %) | | |
| Long antennae | Short antennae | | |
| More sensilla on hind legs | Less sensilla on hind legs | | |
| More frontal hairs | Less frontal hairs | | |
| Less touch receptors | More touch receptors | | |
| | | Shorter sternal hairs | Longer sternal hairs |
| | | Less spermatophores | More spermatophores |
| **Physiological differences** | | | |
| Long-lived | Short-lived | Long-lived | Short-lived |
| Long maturation times | Short maturation times | Short maturation times | Long maturation times |
| Lower $O_2$ consumption | Higher $O_2$ consumption | Lower $O_2$ consumption | Higher $O_2$ consumption |
| Lower heartbeat frequency | Higher heartbeat frequency | Lower heartbeat frequency | Higher heartbeat frequency |
| Lower fat content | Higher fat content | Lower fat content | Higher fat content |
| Lower carbohydrate content | Higher carbohydrate | Lower carbohydrate content | Higher carbohydrate |
| Lower spontaneous activity of TCD in the light[3] | Higher spontaneous activity of TCD in the light | | |
| No difference between activity of TCG in light and dark[4] | Higher spontaneous activity of TCG in the dark | | |

| **Molecular differences** | | | |
|---|---|---|---|
| Lower SGPP1-4 expression[5] | Higher SGPP1-4 expression | Higher LMPP2 expression[6] | Lower LMPP2 expression |
| No difference in octopamine levels | No difference in octopamine levels | Higher octopamine levels | Lower octopamine levels |
| Lower JH titer[7](debated) | Higher JH titer (debated) | Higher overall JH biosynthesis | Lower overall JH biosynthesis |
| Low ecdysteroid level in eggs | High ecdysteroid level in eggs | | |
| High ecdysteroid levels in haemolymphe | Low ecdysteroid levels in haemolymphe | | |
| No phenylacetonitrile production | Phenylacetonitrile production | | |
| Low yellow protein (YP) levels | High YP levels in adult males | | |
| Low carotenoid/biliverdin ratio | High carotenoid/biliverdin ratio | | |
| Low phase-related peptide (PRP) level | High PRP level | | |
| Low levels of PRP in eggs | High levels of PRP in eggs | | |
| Slower corazonin breakdown | Faster corazonin breakdown | | |
| High expression of NPP1-2[8] | Low expression of NPP1-2 | | |
| Low expression of NPP3-4[8] | High expression of NPP3-4 | | |
| Higher levels of 8 neurochemicals: Arg, Asp, Glu, Gly, taurine, GABA, dopamine, and serotonin | Higher levels of 4 neurochemicals: acetylcholine, citrulline, N-acetyldopamine, and tyramine | | |
| Higher levels of 155 metabolites, especially phosphatidylethanol-amines (PEs) and diacyl-glycerols (DGs) | Higher levels of 164 metabolites, especially carnitines, lysophosphatidylcholines, several carbohydrates, amino acids, and some DGs | | |

| **Molecular differences (continued)** | | | |
|---|---|---|---|
| Differential expression in 214 genes (long-term solitarious), 114 genes upregulated, including genes involved in energy metabolism, protein synthesis, and antioxidant genes | Differential expression in 214 genes (long-term gregarious), 100 genes upregulated, including heat-shock and immunity genes | Upregulation of genes involved in energy metabolism and protein synthesis | Upregulation of 6 heat-shock protein (HSP) genes |
| | | Lower levels of Neuroparsin A | Higher levels of Neuroparsin A |
| | | Lower OMP levels in mature adults[9] | Higher OMP levels in mature adults |
| | | Higher levels of APRP[10] | Lower levels of APRP[10] |
| | | Lower levels of dopamine | Higher levels of dopamine |
| | | Higher haemolymph levels of lipid, trehalose, and putrescine | Lower haemolymph levels of lipid, trehalose, and putrescine |
| | | Differential expression in 532 genes (2-3 generations), including upregulation of mostly anabolic, biosynthetic, and muscle-specific gene expression | Differential expression in 532 genes (2-3 generations), including very strong upregulation of the JHPH gene family[11] in the head |
| | | | Upregulation of a cluster of synaptic transport components, neurotransmitter and neuro-modulator receptors, neuro-transmitter synthetases, and G protein coupled receptors |
| | | Upregulation of *take out* in antennae at 4$^{th}$ instar | Upregulation of genes for CSPs (chemosensory protein) at 4$^{th}$ instar |

| Molecular differences (continued) | | |
|---|---|---|
| | | Upregulation of genes of general metabolism and catecholamine biosynthesis at 4th instar |
| | Differential expression in 4893 genes in the brain (short-term, 64h), including upregulation of genes in redox biology | Differential expression in 4893 genes in the brain (short-term, 64h), including upregulation of genes in synaptic transmission, carbohydrate metabolism and nucleosome assembly |
| | Higher abundance of longer (> 26-29 nt[12]) small RNAs | Lower abundance of longer (> 26-29 nt) small RNAs |
| | Higher expression levels of micro-RNA 133 | Lower expression levels of micro-RNA 133 |
| | Upregulation of transposon element I in antenna and labial palp at 4th instar | Upregulation of transposon element I in brain and wings at 4th instar |
| | | 45 alternative spliced isoforms between gregarious and solitarious animals |
| | | 90 genes differentially methylated |

| **Behavioural differences** | | | |
|---|---|---|---|
| Flight at night | Flight by day | Flight at night | Flight by day |
| Slow, creeping gait | Rapid, upright gait | Slow, creeping gait | Rapid, upright gait |
| Restricted diet | Generalists | Restricted diet | Generalists |
| Avoid other locusts | Attracted to locusts | Avoid other locusts | Attracted to locusts |
| Relatively sedentary | More active | Relatively sedentary | More active |
| Less intense mating behaviour | More intense mating behaviour | Less intense mating behaviour | More intense mating behaviour |
| Lower grooming frequency | Higher grooming frequency | Lower grooming frequency | Higher grooming frequency |

Similar findings for both species are listed first, followed by differences (italic) and findings that have only be described in one of the species. Abbreviations: [1] F/C (length of femur/ maximum head width), [2] E/F (length of fore-wing/length of femur), [3] TCD (tritocerebral dwarf), [4] TCG (tritocerebral giant), [5] SGPP (*Schistocerca gregaria* pacifastin-related precursor), [6] LMPP (*Locusta migratoria* pacifastin-related precursor), [7] JH (juvenile hormone), [8] NPP (neuroparsin precursor), [9] OMP (ovary maturating parsin), [10] APRP (adipokinetic hormone (AKH) precursor-related peptide), [11] JHPH gene family (juvenile hormone binding protein, hexamerins, prophenoloxidase, and hemocyanins), [12] nt (nucleotides)

## 4.3 Making the switch: initiation of phase transition

To date, no single factor has been identified that can induce the entire set of alterations in a locust during phase transition. However, during the past two decades a number of factors have been identified that are involved.

### 4.3.1 Visual, olfactory and/or mechanosensory information

Two distinct sensory pathways are involved in the onset and continuation of aggregating behaviour: the cerebral pathway, incited by the combined visual and olfactory stimuli, and the thoracic pathway, induced by tactile information (Burrows *et al.* 2011). Stimulating the hind leg, but not other parts of the body, is sufficient for gregarization behaviour to occur (Ellis 1959, Simpson *et al.* 2001). In the Australian plague locust, *Chortoicetes terminifera* (WALKER 1870), stimulation of the antennae, but not the hind legs, is sufficient to elicit a phase transition (Cullen *et al.* 2010). Not only tactile stimulation, but the combined sight and smell of conspecific or heterospecific locusts can induce gregariousness in locust nymphs as well (Roessingh *et al.* 1998, Lester *et al.* 2005). However, it has been reported that certain contact chemicals by themselves can induce gregarization behaviour (Heifetz *et al.* 1996, Heifetz *et al.* 1997, Heifetz *et al.* 1998). For a review on the roles of semiochemicals in locusts, we refer the reader to Hassanali and co-workers (2005). On the other hand, Tanaka and Nishide report that sight of moving animals is sufficient to induce darkening in desert locusts, whereas odour had barely any effect (Tanaka and Nishide 2012).

Tactile information through the antennae reflecting the degree of crowding experienced by the mother directly influences the colour of hatchlings in *S. gregaria* and *L. migratoria*, arguing for a maternal factor (Maeno *et al.* 2011). Most likely, an alkylated L-dopa analogue recently isolated from egg foam that could induce gregarious behaviour in nymphs hatched from treated eggs deposited by solitarious females fulfils this role (Miller *et al.* 2008, Islam 2013).

### 4.3.2 Neuroendocrine control of phase transition: corazonin, juvenile hormone, serotonin, and dopamine

Although the concentration of several potential neurochemicals differs between solitarious and gregarious locusts, only serotonin shows a dramatic transient increase within hours of crowding (Rogers *et al.* 2004). Moreover, injection of serotonin (and analogues) is sufficient to induce gregariousness in both *S. gregaria* and *L. migratoria*, whereas injecting serotonin antagonists inhibits tactile-induced phase transition

(Anstey *et al.* 2009, Ma *et al.* 2011). These observations establish serotonin as an important regulatory agent in phase transition, an effect that is most likely mediated through protein kinase A signalling (Ott *et al.* 2012). We note that other researchers report no influence on attraction/avoidance behaviour by serotonin (Tanaka and Nishide 2013) or on darkening of hatchlings (Maeno *et al.* 2011) in *S. gregaria*. However, in the latter experiments serotonin was injected through the abdominal sternites in the thorax instead of directly into the thoracic ganglia, which could account for the difference in results.

Surprisingly, injection of serotonin or 5-HTR agonists into the head cavity of gregarious *L. migratoria* nymphs shifts their behaviour towards solitariousness (Guo *et al.* 2013a), suggesting serotonin is involved in both the gregarization and solitarization decision. These results correspond well with earlier observations where serotonin displays a peak in the optic lobes (located in the head cavity) shortly after isolation, while a similar peak is seen in the thoracic ganglion when these individuals are reaggregated (Rogers *et al.* 2004).

In addition to serotonin, pharmacological and transcriptional silencing experiments identified dopamine as another potent gregarization factor in the migratory locust, driving both behavioural transition and melanin deposition (Ma *et al.* 2011).

Tanaka 2006 has postulated that juvenile hormone (JH) in conjunction with corazonin can account for body colour polyphenism, both in the desert and migratory locusts. Corazonin, an undecapeptide released from the *corpora cardiaca* (CC), causes darkening of the locust body colour (Vandersmissen *et al.* 2006), whereas JH and JH analogues induce the green body colour in solitarious forms (Hasegawa and Tanaka 1994). Besides instigating dark body colouring, corazonin induces a more convex pronotum in isolated-reared locusts (Tanaka *et al*. 2002), changes body aspect ratios towards gregarious animals (Hoste *et al.* 2002, Tanaka *et al.* 2002, Breuer *et al.* 2003, Maeno *et al.* 2004), and reduces the number of antennal sensilla to that of crowd-reared locusts (Maeno and Tanaka 2004, Yamamoto-Kihara *et al.* 2004). However, corazonin cannot induce gregarious behaviour (Hoste *et al.* 2003) or the darkening of offspring of solitarious individuals (Tanaka 2001).

## *4.4 Comparing solitarious and gregarious phases: molecular differences derived from –omics data*

The recent publication of the first draft of the 6.5 Gbp genome sequence of the migratory locust (Wang *et al.* 2014) marks a crucial milestone that should greatly aid researchers in their quest to unravel the molecular foundations underlying locust phase change. Earlier -omics approaches have been instrumental in gaining new insights into

the physiological transformation that differentiates the solitarious locust from its gregarious phase, and will be summarized in this section.

### 4.4.1 Proteomics and peptidomics

*S. gregaria* pacifastin-like precursors (SGPP1-4), peptides with still unknown functions, display a strong phase-dependent transcription in brain and fat body, suggesting a physiological role in phase transition (Simonet *et al.* 2004, Simonet *et al.* 2005). Similarly, LMPP2 has a differential expression between both phases (Kang *et al.* 2004). Neuroparsin precursors (NPP) from the desert locust (*Scg-NPP1-4)* similarly show a very distinct phase-dependent expression (Claeys *et al.* 2005, Claeys *et al.* 2006b). Interestingly, NPP transcription is induced upon injection with JH or 20-hydroxyecdysone (Claeys *et al.* 2006a).

In *L. migratoria,* higher levels of Neuroparsin A (NP-A) and ovary maturating parsin (Lom-OMP) were found in the CC of crowded locusts. In contrast, the concentration of adipokinetic hormone (AKH) precursor-related peptide (APRP) was decreased (Ayali *et al.* 1996). APRPs are by-products of AKH synthesis with an as yet unknown functional role (Hatle and Spring 1999, Baggerman *et al.* 2002). Neuropeptide profiles of CC and haemolymph confirmed phase-differentiating levels of AKH I and II and APRPs in *S. gregaria,* but strong sex- and age-dependent concentration differences were also observed (Clynen *et al.* 2002).

The most enigmatic factor is the phase-related peptide (PRP) (Clynen *et al.* 2002), a 6 kDa peptide present in much higher concentrations in the haemolymph of gregarious locusts (up to 0.1 mM) than in solitarious locusts. Interestingly, upon isolation of crowd-reared locusts this peptide shows a progressive decrease in concentration spanning multiple generations. This finding represents another clear manifestation of the epigenetic aspects of locust phase differentiation and maintenance. Higher concentrations of this peptide are also found in eggs of gregarious than of solitarious *S. gregaria* (Rahman *et al.* 2002, Rahman *et al.* 2003a).

### 4.4.2 Metabolomics

Comparison of the metabolic profiles of haemolymph between solitary and crowd-reared *S. gregaria* using $^{1}$H NMR spectroscopy revealed over 20 differentially abundant metabolites (Lenz *et al.* 2001). Most notably, crowd-reared insects showed much lower levels of lipid and trehalose, as well as the polyamine putrescine, a breakdown product of amino acids.

More recently, HPLC-GC/MS-based metabolic profiles of solitary and gregarious *L. migratoria* haemolymph were analysed over the time course of phase transition (Wu *et al.* 2012). Over 45 % (319) of the detected metabolites differed between the two phases with multiple lipids, carbohydrates, amino acids, and carnitines showing the most prominent changes. Interestingly, the genetic or pharmacological manipulation of acyl- and acetylcarnitine levels induces both metabolic and behavioural changes associated with phase transition. Since carnitine is responsible for the transport of fatty acids into the mitochondrial matrix prior to their degradation, this could reflect increased energy requirements in gregarious locusts. Alteration in energy metabolism, membrane fluidity, and lipid-mediated cell signalling are all potential avenues by which changes in lipid metabolism could be implicated in the phase transition.

### 4.4.3 Transcriptomics

In an early attempt to investigate differential expression in phase transition, Rahman and co-workers used differential display RT-PCR. They found one gene with higher expression in gregarious desert locusts (resembling *Drosophila* SPARC) and one unidentified gene with higher expression in solitary locusts (Rahman *et al.* 2003b). The construction of EST databases since greatly facilitated locust research. In 2004, a high-coverage EST data set (76,012 ESTs) of the whole body, head, hind legs, and midgut tissue of *L. migratoria* hoppers was generated (Kang *et al.* 2004). A general repression of (mostly anabolic, biosynthetic, and muscle-specific) gene expression was observed in the hind leg, midgut, and head tissue of gregarious compared to solitarious animals, an observation consistent with their weaker leaping ability. The relative expression pattern in the head of gregarious animals was characterized by the strong upregulation of a functionally diverse set of genes. Most notably, very strong activation of the JHPH (juvenile hormone binding protein, hexamerins, prophenoloxidase, and hemocyanins) gene family was observed in the head of gregarious locusts. These findings suggest extensive expressional changes in nerve cells that could reflect how hormonal signals govern the phase state transition. The same group also described elevated transcript levels of 6 heat-shock protein (HSP) genes in gregarious locusts compared to solitary locusts (Wang *et al.* 2007). This activation of HSP expression likely reflects a stress-response triggered by increased population density. This increase in expression of genes related to stress was consistent with the findings of Badisco and co-workers. They detected 214 differentially expressed genes (Badisco *et al.* 2011b) in an EST database (34,672 ESTs) generated from the central nervous system of both phases of the desert locust (Badisco *et al.* 2011a). The upregulation of heat-shock and immunity genes in gregarious locusts seems to offer protection from the acute detrimental effects of

crowding. At the same time a reduction in expression of genes in energy metabolism and protein synthesis is observed. This reduction of metabolism and biosynthesis in gregarious locusts was also found in migratory locusts by comparing the transcriptome (72,977 sequences) of developing solitary and gregarious locusts (Chen *et al.* 2010). Taken together, these data clearly suggest that gregarious locusts are associated with a physiological stress state (Boerjan *et al.* 2010). Conversely, expression of antioxidant genes is repressed in gregarious desert locusts, suggesting increased susceptibility to oxidative stress (Badisco *et al.* 2011b). In gregarious migratory locusts a cluster of synaptic transport components, neurotransmitter and neuromodulator receptors, neurotransmitter synthetases, and G protein-coupled receptors (GPCRs) was also prominently upregulated, pointing to an important neuromodulary aspect in phase transition (Chen *et al.* 2010). In addition, it was found that fourth instar locusts displayed the most divergence between polymorphic states. This was confirmed by micro-array studies, which illustrated that the top pathways affected in gregarious nymphs at fourth stage were involved in general metabolism and catecholamine biosynthesis (Ma *et al.* 2011).

Another micro-array study on fourth instar migratory locust during phase transition revealed differential expression in chemosensory proteins (CSPs) and *takeout* proteins (Guo *et al.* 2011). Genes of these families were found highly expressed in peripheral tissues (sensilla, antennae, labial palps, wings, and hind legs) but not internal tissues. RNAi against chemosensory proteins increased repulsion in gregarious, whereas knockdown of one *takeout* gene (*LmigT01*) increased attraction behaviour in solitary fourth instar nymphs most likely by altering the peripheral olfactory sensitivity.

Large-scale transcriptome analysis revealed 105 retro-elements in the migratory locust, some of which show a differential expression pattern between the solitarious and gregarious phase at the fifth instar and in adults (Jiang *et al.* 2012). The developmental and tissue specific expression pattern of a single class I transposon element was also highly different in gregarious compared to solitarious locusts (Guo *et al.* 2010). These observations have prompted the Kang lab to propose a regulatory role for these transposon elements in the phase transition of migratory locusts (Jiang *et al.* 2012). Interestingly, the degree of genome methylation constitutes an important factor determining transcriptional activity of (retro)transposons and suggests one possible role for genome methylation to modulate phenotypic plasticity in insects (Slotkin and Martienssen 2007).

A recent trancriptome analysis of brain tissue of locusts experiencing short-term (64 hours) gregarization and solitarization revealed a staggering 4893 differentially expressed genes (28 % of the transcriptome) in both processes (Wang *et al.* 2014). Increased expression of genes in synaptic transmission, carbohydrate metabolism and

nucleosome assembly seem to point towards increased neuronal activity during locust crowding, while at the same time lowered expression of genes in redox biology suggests suppression of antioxidative responses in the CNS. The same authors also identified 45 genes where alternative splicing (AS) results in differential isoform expression between phases. A common theme in the transcriptome, AS and methylome datasets (see below) is the differential expression (and methylation) of cytoskeletal/microtubular genes, likely reflecting the neuronal plasticity accompanying the behavioural changes upon phase transition.

## *4.5 Epigenetic mechanisms in invertebrates*

### 4.5.1 Histones in insect epigenetics

Next to DNA methylation and demethylation processes, reversible posttranslational modifications (PTM) of histone proteins are the best-studied elements of epigenetic mechanisms. More than 160 histone modifications, *e.g.* methylation, acetylation, and phosphorylation (Bannister and Kouzarides 2011, Suganuma and Workman 2011, Tan *et al.* 2011), alter chromatine structure and density and hence the accessibility of DNA, which influences transcription rates ("histone code") (Strahl and Allis 2000, Jenuwein and Allis 2001). Histone modifications and chromatine states have been intensively studied in *D. melanogaster* (Filion *et al.* 2010, Kharchenko *et al.* 2011, Negre *et al.* 2011). Less is known about the role of histone PTMs in other insects. However, Nanty and colleagues showed that patterns of histone PTMs are largely conserved between invertebrate species and can therefore be predicted for different taxa (Nanty *et al.* 2011). Indeed, DNA methylation and histone modifications seem to work together, if not redundantly, to influence gene expression patterns (Hunt *et al.* 2013b, Hunt *et al.* 2013a).

Several histone PTMs have been characterized in honeybees (Dickman *et al.* 2013). To date, the best evidence for the involvement of histone modifications in insect polyphenism comes from the carpenter ant *Camponotus floridanus* (BUCKLEY 1866), where the pattern of acetylation of lysine 27 at histone 3 (H3K27ac) differs between males, major and minor workers (Simola *et al.* 2013).

### 4.5.2 Non-coding RNA and other epigenetic mechanisms

While DNA methylation and histone modifications are the most prominent epigenetic mechanisms studied to date, non-coding (nc) RNA and heritable protein alteration recently got increased attention. Altered protein conformations transmittable to subsequent generations have been studied in yeasts (Halfmann *et al.* 2010, Halfmann

and Lindquist 2010, Halfmann *et al.* 2012). Similar mechanisms have been suggested for *Drosophila*, but have yet to be proven (Tariq *et al.* 2013).

Non-coding RNAs may have a more prominent role in epigenetic mechanisms than previously thought. New classes of ncRNA, including short interfering RNA (siRNA), microRNA (miRNA), PIWI-interacting RNA (piRNA), and long ncRNA (lncRNA), have varying roles in gene regulation (Jacquier 2009, Moazed 2009, Pauli *et al.* 2011). In honeybees and ants, for instance, miRNAs have been implicated in (temporal) caste differences (Behura and Whitfield 2010, Bonasio *et al.* 2010, Greenberg *et al.* 2012, Liu *et al.* 2012, Guo *et al.* 2013b).

## 4.6 Epigenetics in life phase transitions

Epigenetic mechanisms could play a crucial role in life phase transitions in insects. In the honeybee, DNA methylation and histone modifications mark two important processes: (1) the irreversible differentiation of a female larvae into a queen or worker phenotype (Kucharski *et al.* 2008) and (2) for worker bees the reversible shift from a temporal nurse subcaste to the forager subcaste (Herb *et al.* 2012; Lockett *et al*. 2012). This has also been studied in several ant and wasp species (Bonasio *et al.* 2012, Simola *et al.* 2013, Weiner *et al.* 2013, Bonasio 2014, Bonasio 2015). The differentiation into a queen or a worker has dramatic consequences: a honeybee queen lives several years, is much larger, highly fertile, and differs also in many more morphological traits and behavioural suits from her sisters who developed into a worker and have a life expectancy of only a few weeks Winston 1987.

Despite the overwhelming indications for an important role of epigenetics in the regulation of phase transitions in insects, the direct proof is relatively limited. The best-known example is the induction of queen-like phenotypes in honeybees, *Apis mellifera*, by downregulating of Dnmt3 (Kucharski *et al.* 2008, Li-Byarlay *et al.* 2013). In the buff-tailed bumble-bee, *Bombus terrestris* LINNAEUS 1758, experimental alteration of DNA methylation by feeding 5-aza-20-deoxycytidine (Decitabine) renders queenless worker bees more aggressive and more fertile (Amarasinghe *et al.* 2014). However, the drug treatment was only successful in callow workers (*i.e.* younger than 1 day), whereas older workers are also able to activate their ovaries.

In the crustacean water flea *Daphnia magna* STRAUS 1820, exposure to 5-azacytidine reduced overall DNA methylation as well as body length (Vandegehuchte *et al.* 2010). Interestingly, this hypomethylation pattern was transferred to two subsequent generations that were not exposed to the drug, demonstrating transgenerational epigenetic inheritance. These two generations were also shorter, but as yet there is no link established between hypomethylation and body length. However, it should be noted

that 5-azacytidine as well as other nucleoside analogues are not specific DNA methylation inhibitors and also affect other pathways (Gnyszka *et al.* 2013, Poirier *et al.* 2014).

Further evidence for epigenetic inheritance in *Drosophila* and *Daphnia* is reviewed by others (Youngson and Whitelaw 2008), but the mechanisms of this "soft" inheritance are generally not well understood.

## 4.7 Evidence for epigenetics in locusts

### 4.7.1 DNA methylation

To our knowledge, the earliest study on DNA methylation in locusts dates from 1951 (Wyatt 1951), and reported that 0.96 % of all cytosines are methylated in *L. migratoria*. Surprisingly, these early findings were followed by a 60-year gap. In 2011, we showed that, compared to other insects, *S. gregaria* DNA is relatively highly methylated (1.3-1.9 percent of total cytosines, depending on tissue) (Boerjan *et al.* 2011). The *Schistocerca* transcriptome contains transcripts for some of the enzymes belonging to the epigenetic machinery, including a methyl binding protein (MBD), a histone acetyl transferase (HAT), a histone deacetylase (HDAC), and homologues of Dnmt1 and Dnmt2 (Boerjan *et al.* 2011, Falckenhayn *et al.* 2013). Expression levels of Dnmt2 in the metathoracic ganglion change during crowding. Next-generation shotgun bisulfite sequencing to identify the *S. gregaria* methylome confirmed 1.3-1.4 % cytosine methylation, 90 % of which in a CpG context (Falckenhayn *et al.* 2013). The locust genome is higher methylated than most known insect genomes (but less than other Orthoptera: *L. migratoria* (1.6 %) (Wang *et al.* 2014), *Grylloptalpa fossor* SCUDDER 1869 (3 %) (Sarkar *et al.* 1992), *Chorthippus parallelus* (ZETTERSTEDT 1821) (4.06 ± 0.68 %) (Lechner *et al.* 2013). Since the genome of *S. gregaria* has not been sequenced so far, the sequences were mapped against an EST database (Badisco *et al.* 2011a). Out of those that could be mapped, 3.2 % and 3.1 % cytosines for brain and metathoracic ganglia, respectively, are methylated, 97 % of which in a CpG context. This more than two-fold higher methylation level in comparison with the overall methylation pattern suggests that methylation is targeted to exons. In contrast to the honeybee (Lyko *et al.* 2010) and the silkworm (Xiang *et al.* 2010, Zemach *et al.* 2010), but similar to the stick insect (Krauss *et al.* 2009) and *L. migratoria* (Robinson *et al.* 2011, Wang *et al.* 2014), repetitive elements (rDNA, transposons) are also methylated. Genes with a low CpG observed/expected ratio are assumed to have been (historically) methylated in the germline, as methylated cytosines tend to mutate to tyrosines, causing depletion of cytosines over evolutionary timescales (Bird 1980, Duncan and Miller 1980). Indeed, these genes are more methylated in

*L. migratoria*. Twenty percent of the contigs were over 95 % methylated, another 20 % more than 65 %, an unusually high methylation rate and distinct from other invertebrates. However, gene methylation did not correlate with gene expression levels in six of the investigated genes, suggesting that there is no straightforward link between methylation levels and gene expression.

Recently, the early report of DNA methylation in *L. migratoria* (Wyatt 1951) was confirmed by Robinson and co-workers, who found that 1.3 % of the total cytosines are methylated, which is in the same range as *S. gregaria*. Similar to *S. gregaria,* DNA methylation in *L. migratoria* is not restricted to gene bodies, but also targeted to repetitive elements (Robinson *et al.* 2011). In addition, they found transcripts for methyl binding protein (MBD 2/3), Dnmt2, and two copies of Dnmt1 (Robinson *et al.* 2011). Interestingly, genes differentially expressed between the two phases in *L. migratoria* show signs of CpG depletion. The hypermutability of methylated cytosines leads to formation of thymines via deamination. Thus, a depletion of CpGs over time occurs if highly methylated sequences in the germline were affected. The CpG O/E value is the ratio between observed and expected CpGs within a sequence and is a signature of historical DNA methylation in the germline.

In 2014 the genome sequence of *L. migatoria* was reported (Wang *et al.* 2014) and confirmed the findings of Robinson *et al.* 2011. The *L. migratoria* genome encodes an apparently functional methylation system, containing two copies of Dnmt1 and a single copy of Dnmt2 and 3. Besides the genes reported by Wang and co-workers, we found evidence of additional genes involved in epigenetic mechanisms in the published genome. BLAST searches suggest the presence of at least six HDACs, two HATs, five histone methyltransferases (HMTs), two histone demethylases (HDMs), and one MBD (Supplementary Files 2-3).

A comparative methylome analysis of brain tissues between fourth instar solitarious and gregarious *L. migratoria* (Wang *et al.* 2014) revealed relatively lower and more fluctuating levels of CpG methylation in the coding regions of the genome compared to the whole genome. The ratios of observed/expected CpG levels show a bimodal distribution curve (as in *A. mellifera* and *S. gregaria* (Elango *et al.* 2009, Foret *et al.* 2009, Wang and Leung 2009, Falckenhayn *et al.* 2013)) and suggest historical germline methylation, particularly in the coding regions of the genome. As in *S. gregaria* (Falckenhayn *et al.* 2013), repetitive elements are highly methylated. Introns are more methylated than exons. Ninety genes are differentially methylated (at least 4 differentially methylated CpG sites) in gregarious versus solitarious locusts, including genes involved in cytoskeleton formation. Wang *et al.* suggest that these genes might be involved in synaptic plasticity and point to a, for the phase transition, crucial role of microtubule dynamics control in locust brains.

### 4.7.2 Histone modifications

The role of histone modifications has been less well studied in locusts. Using immunoassays for *S. gregaria*, we found that histone H3 contains phosphorylation (at serine 10 and 28, and threonine 3 and 11, respectively), tri-methylation and acetylation (both at lysine 9 and 27) (B. Boerjan *et al*., unpublished data). Preliminary data suggest that brains of gregarious *S. gregaria* contain more phosphorylated histone H3 than solitarious ones.

### 4.7.3 Non-coding RNA

In migratory locusts, burst expression of retro-elements has been observed in the egg stage, which is thought to be involved in locust development and proposed as a regulatory mechanism in phase transition (Guo *et al.* 2010). The involvement of small non-coding RNAs in *L. migratoria* phase transition was investigated by Wei *et al*., who compared small ncRNA abundances between the gregarious and solitary phase states (Wei *et al.* 2009). The two phases differed strongly in both length distribution and type of small RNAs. Gregarious animals had higher expression of small RNAs below 22 nucleotides, whereas the opposite was true for small RNAs above 22 nucleotides. Gregarious animals also had double the amount of miRNAs, whereas the solitary state expressed higher levels of endo-siRNAs and piRNA-like small RNAs. Moreover, microRNA-133 has been shown to inhibit behavioural aggregation by controlling dopamine synthesis in locusts (Yang *et al.* 2014). All this is in strong support for an epigenetic basis for phase polymorphism.

In summary, increasing evidence points to a pivotal role of epigenetics in controlling phase transitions in locusts, yet definite proof, *i.e.* more than correlational evidence, is still lacking.

## *4.8 Future research directions*

Until recently, evidence for methylation in locusts relied on paper chromatography and photo-spectroscopy (Wyatt 1951), mass spectrometry (Boerjan *et al.* 2011, Lechner *et al.* 2013), and methylation specific restriction enzyme assays (Robinson *et al.* 2011). While these methods have their specific value, single base resolution methylome analyses allow detailed analysis of hypothetical changes in DNA methylation status between the solitary and gregarious phase (Robinson *et al.* 2011). Recently, these analyses have been performed in *L. migratoria* (Wang *et al.* 2014). Parts of a *Schistocerca* methylome have also been characterized (Falckenhayn *et al.* 2013), but, unfortunately, differences between phases were not investigated. Methylome studies to date have been

based on whole organisms or brain tissue. However, it is likely that some epigenetic differences might be concealed by these approaches, as they might be specifically directed to particular brain regions or even cells (Bonasio 2012).

### 4.8.1 Pharmaceutical manipulation of global DNA methylation status

Strong proof for a role of epigenetics in locust phase transition would be the experimental switch between phases in the treated animal and/or its offspring by altering parts of the epigenetic setting, be it DNA methylation, histone modification, ncRNAs, nucleosome positioning, or others. The epigenetic machinery can be experimentally manipulated, *e.g.* by blocking involved enzymes (such as Dnmts or MBDs), or by downregulating the synthesis of these enzymes. In recent years, it became even possible to manipulate DNA methylation and histone modifications at specific sites (see below).

Gene regulation by DNA methylation is a complex matter (not to mention the intricate interactions with histones, their modifications, and ncRNAs), and transitions from one state to another are usually characterized by a dynamic alteration in the methylation pattern. While some genes (or promoters) are being methylated, others are demethylated, and as such the total content of methylated cytosines could be more or less constant. For instance, most cancers are associated with a specific methylation pattern, where tumour suppressor genes are hypermethylated (*i.e.* inactivated), while oncogens are hypomethylated (*i.e.* activated). It is evident that drugs or other experimental methods that aim at a general lower or higher level of DNA methylation are rather crude tools and will likely not be able to mimic such a delicate balance. Indeed, some cancer forms are associated with global hypomethylation, which also might promote metastasis (reviewed in Szyf 2009). A complete demethylation is usually lethal, and Dnmts are often essential during development (*e.g.* Zwier *et al*. 2012).

Drugs like Zebularine (Zhou *et al.* 2002), Azacytidine, and Decitabine (reviewed in Christman 2002) are nucleoside analogues that prevent *de novo* and maintenance methylation by binding covalently to Dnmts when incorporated into DNA. Since these drugs do not actively remove methyl groups, they are only effective where DNA replication takes place, limiting their usage to dividing cells.

Several other compounds that are not incorporated in DNA have been described as Dnmt inhibitors or DNA demethylating agents (reviewed in Szyf 2009), but their mode of action is not yet understood. Mechanisms and enzymes that actively demethylate DNA have been described in mammals and plants, the most prominent being TET (ten-eleven translocation) proteins (reviewed in Kohli and Zhang 2013, Piccolo and Fisher 2014, Wu

and Zhang 2014). Some of these demethylating pathways do not require DNA replication and would therefore be attractive to manipulate post-mitotic cells.

It should be noted that DNA methylation in insects is not necessarily associated with silencing of gene expression; in fact, DNA methylation in insects is highly correlated with steady gene expression and reduced variability in transcript levels (Foret *et al.* 2009, Lyko *et al.* 2010, Zemach *et al.* 2010).

### 4.8.2 Pharmaceutical manipulation of global histone modifications

Besides targeting DNA methylation, another promising avenue would be the manipulation of histone modifications. In humans, several classes of drugs have been developed for treating cancer and psychological disorders that interact with histone modifying enzymes such as histone methyltransferase (HMT), histone demethylase (HDM), histone acetyltransferase (HAT), or histone deacetylase (HDAC) (reviewed in Szyf 2009, Grayson *et al.* 2010). Some of these enzymes are widely conserved, and could thus serve as targets for pharmaceutics. Interestingly, HDAC inhibitors (HDACis) will not affect the whole genome, as both HDACs and HATs appear to be specific for certain sequences (reviewed in Szyf 2009), which offers the opportunity to target a subset of genes. Some HDACis also induce active DNA demethylation independent of DNA replication, which circumvents the disadvantages of drugs based on nucleoside analogues that are restricted to replicating DNA.

Similarly, HMT inhibitors will prevent the methylation of specific histones, which in turn prevent DNA methylation. On the other hand, inhibition of histone demethylases would shift the balance between methylation and demethylation towards higher methylated histones. Histone (de)methylating enzymes are also specific, with several dozen specimen in several families described so far (reviewed in Hojfeldt *et al.* 2013). So far, however, there are no reports of active demethylation processes in insects. While some drugs have been shown to work in humans and *Drosophila* alike (*e.g.* Greiner *et al.* 2005, but see also Cherblanc *et al.* 2013), for some it remains to be evaluated whether they are also effective in other insects.

### 4.8.3 Analyses of DNA and histone modifications

Recent technologies allow to map different DNA modifications (methylation, hydroxymethylation, formylation, carboxylation) at single-base resolution (Booth *et al.* 2012, Raiber *et al.* 2012, Yu *et al.* 2012, Song *et al.* 2013), and we are only beginning to grasp the significance of these unusual nucleobases.

Various methods are available to map DNA methylations, each with their specific advantages and shortcomings (Bock *et al.* 2010, Harris *et al.* 2010, Laird 2010, Nagarajan *et al.* 2013, Umer and Herceg 2013, Mensaert *et al.* 2014). Wang *et al.* 2014 used reduced representational bisulfite sequencing (RRBS) to map methylated CpG in brains of solitarious and gregarious locusts. This approach allows single-base resolution and absolute quantification of (hydroxy)methylation (albeit with imperfect quantification (Harris *et al.* 2010)), but is biased towards CpG rich regions (Bock *et al.* 2010, Harris *et al.* 2010) and by the choice of the used restriction enzyme (Deng *et al.* 2009) and size selection of fragments (Bock *et al.* 2010). Both, RRBS and whole genome bisulfite sequencing (WGBS), may contain artifacts due to the bisulfite conversion process and subsequent PCR bias.

Studies of DNA modifications should be complemented by analyses of histone modifications where possible, *e.g.* by ChIP-Seq (Nagarajan *et al.* 2013, Rivera and Ren 2013). A comparison of the epigenomes of the two phases between *L. migratoria* and *S. gregaria* will reveal the extent of common mechanisms as well as potentially private modifications. Epigenetic changes that are shared between the two species should be prioritized in investigating their role in phase transition. It will be interesting to unravel how the different epigenetic pathways in these two species interact to bring about the phenomenon of phase transition.

### 4.8.4 Genome and epigenome editing

More specific tools to manipulate particular epigenetic signatures will have to be developed, *e.g.* "site-specific epimutagenesis", as suggested by Bonasio 2012. One strategy might be to target genes with a role in epigenetics using RNAi. Due to the robust systemic RNAi mechanism in locusts (Wynant *et al.* 2012), knockdown of target genes throughout the insect can easily be achieved by injecting dsRNA into the haemolymph. While RNAi has been successfully used for several years in locusts, it has one major drawback: it rarely induces a complete loss of function of the targeted gene. Innovative site-specific genomic engineering tools are currently being explored. For instance, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or CRISPR-Cas (clustered regularly interspaced short palindromic repeat- CRISPR-associated proteins) could be used to specifically introduce mutations (indels, insertions and deletions) in target genes with roles in epigenetics, such as HATs and HDACs. CRISPR-Cas systems could even be used to edit several genes at once (Jao *et al.* 2013, Li *et al.* 2013b, Wang *et al.* 2013). Since HATs and HDACs are substrate specific, this would only affect a subset of histones. Alternatively, methylated DNA sequences of interest

might be specifically excised and replaced by unmethylated DNA, or vice-versa (Ramalingam *et al.* 2013).

Even more intriguing was the suggestion that methylating and demethylating enzymes as well as histone modifying enzymes (*e.g.*, HMT, HAT) might be directed to specific DNA sequences of interest using TALE or CRISPR-Cas (Bonasio 2012, Gaj *et al.* 2013, Rivera and Ren 2013). This would allow to specifically (de)methylate DNA and histones, thereby enabling to manipulate epigenomes with high precision. This can be accomplished by using a catalytically inactive (or "dead") version of Cas9, called dCas9, fused to an effector domain that will carry out its function (Sander and Joung 2014). While the CRISPR-Cas systems have already been shown to work in diverse organisms, such as bacteria, plants, vertebrates, and insects, it has not yet been employed in locusts.

Recently, several groups succeeded in using TALE to selectively demethylate DNA (Maeder *et al.* 2013) and histones (Mendenhall *et al.* 2013). Konermann *et al.* 2013 were able to switch histone deacetylation and methylation on and off in a time and space specific manner using light-inducible transcriptional effectors (LITEs) in a combination of TALEs and optogenetic methods. This method is highly versatile, as it allows to combine different DNA sequence recognition systems (*e.g.* zinc-finger, TALE) with various "switches" (*e.g.* light or chemical induced) and effectors (*e.g.* HDACs, HMTs). These manipulations would allow functional analyses of specific sites in the epigenome.

However, to date, locusts have not yet been genetically or epigenetically modified. One of the challenges will be to deliver the modified enzymes into cells of interest (Gaj *et al.* 2013). Interestingly, some ZFNs can be delivered directly as proteins across the cell membrane (Gaj *et al.* 2012), but this is unlikely to work for the complex LITEs, which have been delivered by viral vectors (Konermann *et al.* 2013). In *D. melanogaster*, injection of cas9 - as DNA, RNA, or protein - into the embryo allowed genetic manipulation that was transmitted to the offspring through the germline in 25-100 % of the cases, depending on the method and genes targeted (*e.g.* Bassett and Liu 2014, Lee *et al.* 2014, Port *et al.* 2014). Given the ready use of RNAi in locusts (Wynant *et al.* 2012), it is possible that a similar approach will work in locusts as well. If successful, this would open unprecedented opportunities for functional genomics and epigenomics, circumpassing the drawbacks of RNAi and pharmaceutical approaches.

## 4.9 Conclusions

Future research will reveal the mechanisms of epigenetic control of locust phase transition. Additional insights are to be expected from comparisons with the Australian plague locust *Chortoicetes terminifera*, where phase characteristics can change within 72 h in both directions and change abrupt between generations. Given the advent of new

and exciting techniques that allow the specific manipulation of genes and proteins, and the analysis of single cells, these are excellent times to study the molecular mechanics of phase polyphenism in locusts.

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Chapter Five[28,29]

# 5 Life-prolonging measures for a dead theory?

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Selman and colleagues (2012) recently discussed the status quo of the oxidative stress theory of aging (OSTA) and how it links to life history evolution. In short, OSTA posits that reactive chemical agents damage cellular structures, including DNA, and that these damages cause aging and ultimately death (Selman *et al.* 2012). According to the disposable soma theory, there is a trade-off between soma maintenance and reproduction (Kirkwood 1977). Short-lived organisms are predicted to neglect oxidative damage, whereas longer-lived organisms should trade off fertility and protection against oxidative damage. However, there is increasing evidence that reactive oxygen species (ROS) are not causally linked with aging (Pérez *et al.* 2009). Selman et al. (2012) agree, yet argue that there might still be a role of oxidative damage in shaping life history and suggest that this would only be measureable in the wild. We disagree with this proposition, based on the aforementioned published data (*e.g.* Buffenstein 2008, Buffenstein *et al.* 2008, Voituron *et al.* 2011), and argue that controlled laboratory experiments are better suited to test the hypothesized link between oxidative damage and life history evolution.

Oxidative damage per se seems to have a little influence on longevity (Buffenstein 2008), and only severe damage, such as that caused by high concentrations of paraquat, will reduce life-span (Fujii *et al.* 2005).

What, then, are the functions of antioxidative mechanisms, if they do not contribute to longevity in the way it has been assumed in the past? To what extent could they shape life history traits, and how? Unfortunately, Selman and coauthors (2012) do not suggest alternative modes of action, which life history decisions, specifically, could be affected, and which mechanisms might be involved. A relatively new hypothesis is (mito)hormesis, which suggests that naturally occurring low concentrations of ROS function as signaling molecules in longevity pathways but are not the cause of aging (Ristow and Zarse 2010).

Should we, maybe, discuss “health span” rather than life-span? After all, senescent individuals in the wild seldom die peacefully of old age but rather suffer from an associated increased mortality risk (but see Baudisch and Vaupel 2012). It is this survival rate or, more precisely, its associated inclusive fitness that is selected to be maximized. If ROS have a negative effect on longevity and reproductive success, one would predict that individuals with a high fitness should have low levels of ROS. To our knowledge, this has not yet been tested.

Sceptics have raised doubts about the validity of experiments based on small, short-lived organisms in the laboratory. They argue that even if oxidative damage per se does not influence longevity under ideal conditions, it might significantly reduce survival when faced, *e.g.*, with competitors, predators, limited food resources, or diseases. This might be mediated, for instance, by a reduced immune function and decreased sensing

or fighting abilities. However, several examples of long-lived animals in the wild that falsify the OSTA are known (*e.g.* Buffenstein 2008, Buffenstein *et al.* 2008, Voituron *et al.* 2011). While we agree that more data are needed, we propose that these should not be gathered in the wild. Rather, we suggest that experimentation in laboratories or under closely monitored seminatural conditions would yield the most conclusive results. Controlled settings allow for systematic manipulation and evaluation of the factors involved, including genetic diversity. In addition, relevant parameters can be repeatedly assessed in the same individuals. To evaluate the impact of suboptimal conditions on oxidative stress and aging, harsh experimental treatment might be needed, such as malnutrition, exposure to parasites and diseases, extreme temperatures, or social stress. Evidently, such experiments implicate considerable ethical issues that require a sensible ethical debate.

To address the abovementioned concerns, it might be advisable to use larger animals, such as guinea pigs and rabbits. Instead of studying lab-raised zebra finches where artificial selection for fecundity might have altered traits involved in aging, recently, wild-caught birds should be investigated (compare Harper 2008). Additional insights from nonstandard model organisms, such as budgerigars, other birds (Holmes 2004), or fish (Gerhard 2007), will also prove valuable. Potentially, research on semi-wild populations of goats, sheep, deer, or fish could add to the understanding of patterns of oxidative damage and life history decisions.

Currently, it seems that nothing is sure anymore in aging research, and therefore, it is maybe the right time to take leave of dear held theories and develop new approaches to answer the questions raised by Selman et al. (2012).

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Chapter seven

# 6 General Discussion and Future Perspectives

## *6.1 Egg marking pheromone in honeybees*

Despite long investigations, the egg marking signal in honeybees remains enigmatic. In chapter 2 of this thesis (Peptides mark the difference between eggs of queens and workers in honey bees), we provide evidence that a hitherto neglected class of chemicals, namely peptides, could serve the discrimination between QLE and WLE. Interestingly, we found that WLE are coated with more venom components than QLE. Potentially, these cues are used by workers to detect WLE. This would be in line with observations by Martin *et al.* 2005b who found that QLE are removed faster when they had been treated with (apolar) extracts of worker setosa membrane, if the solvent (hexane) would have dissolved some peptides. This would suggest that WLE are actually labelled as "remove me", in addition to lacking the enigmatic queen signal. One could expect that workers should be selected to mimic QLE and thus reduce any suspicious signs of worker origin. However, there is likely a strong selection pressure that selects for powerful venom, and thus workers are likely constraint in reducing the amount of venom they produce and store. If venom really would be a revealing signal of WLE, this might explain why WLE of colonies that have been queenless for a longer period (months) become more acceptable (Martin *et al.* 2005a), since older workers also have less venom (Owen 1983, Owen and Pfaff 1995). Taken together, this might suggest the existence of two signals: a transferable queen signal (WLE placed next to QLE (Martin *et al.* 2005b), WLE previously touched by QLE (Ratnieks 1992), and WLE that touched the queen's sting (Ratnieks 1995) all are longer protected against removal), and a transferable worker signal (QLE treated with extracts of worker setosa are faster removed (Martin *et al.* 2005b)). The hypothesis that venom components are part of the signal is also supported by the observation that WLE become more acceptable after wiping a WLE on the queen's sting shaft and sting sheats (Ratnieks 1995).

Since eggs of anarchistic workers (Oldroyd and Ratnieks 2000, Martin *et al.* 2004a), parasitic Cape honeybees (Martin *et al.* 2002a), and WLE of colonies that have been queenless for a long time (Martin *et al.* 2005a) have higher survival rates, it would be interesting to test whether these WLE also have more queen-like peptide profiles.

However, while we presented promising results, it is not sufficient to identify potential candidates, we need a bioassay to validate whether these compounds are

effectively used. The case is further complicated by the potential existence of a complex signal, *i.e.* one of multiple components, as is the case of the honeybee queen pheromone (Caliari Oliveira *et al.* 2015, Oi *et al.* 2015a). Special difficulties arise with proteins and peptides, as their identification is often cumbersome, and despite the presence of good-quality mass spectra remain often unidentified (Chan *et al.* 2011, Michalski *et al.* 2011, McAfee *et al.* 2015). Thus, we cannot exclude that we have not identified the true queen signal. Indeed, with our focus on proteinaceous components, we might have neglected other polar components.

The most convincing evidence would be if WLE camouflaged with peptides found on QLE would not be policed in discriminatory colonies. However, bioassays to test this are currently lacking. These tests should aim at transferring polar compounds from QLE to WLE and see whether this would make WLE more acceptable. Rubbing WLE against QLE slowed down their removal (Ratnieks 1992). Therefore, extracts of QLE with polar solvents (*e.g.* water, methanol, acetonitrile, dimethylsulfoxid (DMSO)) could serve as source for peptides. These extracts might be concentrated using standard techniques, *e.g.* lyophylised and resuspended, followed by ziptipping (*e.g.* Rappsilber *et al.* 2007). Technically, the transfer of peptides might be difficult to accomplish, because the polar solvents rather form droplets than coating the hydrophobic egg. It has been suggested that spraying the component could circumvent this challenge (Tom Wenseleers, personal communication; compare Katzav-Gozansky *et al.* 2001). Alternatively, submerging the egg in a solution might work. If the egg is allowed to dry in between, several layers might be added on the eggshell to increase the amount of peptides deposited. For all these methods, the eggs must be allowed to dry before they are inserted into the test colony, while at the same time it must be avoided that they desiccate. As a control, QLE and WLE should be treated with the same solvent but without the peptides. These egg types should then be introduced into a discriminatory colony, while a subset of treated eggs should be allowed to hatch in an incubator to control whether the treatment had affected the viability. Ideally, with QLE-extract treated WLE would survive as long as treated and untreated QLE, whereas solvent-treated WLE should survive as long as untreated WLE. In a last step, synthetic peptides applied to WLE should make them more acceptable.

Pirk *et al.* 2007b suggested that honeybees also discriminate against eggs from non-nestmates. While this has not yet been observed in the European subspecies of *Apis mellifera*, it is possible that egg-removal also depends on colony effects (*e.g.* data in Ratnieks and Visscher 1989). Our data (chapter 2, Peptides mark the difference between eggs of queens and workers in honey bees) suggest that peptide profiles are not only caste specific, but also colony specific, making this hypothesis worthwhile to investigate. The experiments are straightforward and require little more than a couple of unrelated

and related queenright honeybee colonies and the patience to transfer hundreds of eggs with a Taber forceps (Taber 1961). Colonies should then receive transferred eggs from their own queen, a related queen (sister, or daughter), and several unrelated queens, and survival after 2 h and 24 h should be monitored (Martin *et al.* 2005b).

Jakob Wegener had suggested that WLE are more prone to desiccation than QLE (Wegener 2009, Wegener *et al.* 2010). Potentially, this might be due to a thinner cuticula, and I suggest to analyse the cuticula of QLE and WLE by light microscopy and transmission electron microscopy in the future[30].

## 6.2 Role of peptides for signalling and immunity in (social) insects

Additionally, it would be of interest whether peptides also play a role in other social insects. Peptides have been ruled out in nest mate recognition in the paper wasp *Polistes dominula* (Bruschini *et al.* 2011), but are used to mark hibernation sides (Turillazzi *et al.* 2006a, Turillazzi *et al.* 2006b) and Dapporto *et al.* 2008 reported that queen and workers differ in their polar cuticular profile (likely of proteinaceous nature). Therefore, it seems possible that also egg recognition is partly mediated by peptides and/or proteins. In first tests, I failed to detect peptides on eggs in the buff-tailed bumblebee *Bombus terrestris*; additional extracts, as well as egg extracts of the common wasp *Vespula vulgaris* and the German wasp *V. germanica* have not been studied yet and await analysis (despite Perry 1996, Perry 2012). It would be interesting to do a comparative study on polistine, stenogastrine and vespine wasps, together with bumblebees and stingless bees. Such a study should aim at egg and larval stages to investigate how widespread peptides occur in (social) insects, and to test whether they could be used as discriminatory signal. Additionally, both nesting material and cuticles of workers should be investigated, similar to studies showing an increase in the strength of antibiotic activity and social immune system with increasing social complexity, as found in bees (Stow *et al.* 2007), thrips (Turnbull *et al.* 2011), and wasps (Hoggard *et al.* 2011). While there was no evidence for venom being applied to the nests of solitary or facultative eusocial wasps (Stenogastrinae) (Baracchi *et al*. 2012), this has not yet been tested for the more advanced vespine wasps (Vespinae), eusocial aphids (*Pemphigus*), or eusocial bees like bumblebees (*Bombus*), stingless bees (Meliponini), or some sweat bees (*e.g. Halictus*, *Lasioglossum*).

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[30] I acknowledge the valuable help of An Vandoren and discussions with Dr. Jakob Wegener and Prof. Dr. Johan Billen.

## 6.3 Physiology and genetics of policing: peptidomics, proteomics, transcriptomics

We have provided evidence for specialisation on worker policing in the honeybee, *Apis mellifera* (chapter 3, Individual and patriline specialisation in policing behaviour in the European honeybee, *Apis mellifera*). Individuals specialise in policing, where some individuals remove a large proportion of eggs, whereas most bees remove no or only a few eggs. Additionally, there is specialisation in patrilines, with some patrilines removing more eggs than expected, based on the size of the patriline, and others not removing any eggs, indicating a heritable component to policing behaviour. Conversely, we found no evidence for age specialisation, *i.e.* policing workers differed not significantly from non-policing workers. This indicates that age is not a major factor in determining which individuals are policing, unlike other tasks as *e.g.* foraging.

Future research should aim at resolving the following questions:

(1) Is policing behaviour related to other behaviours, *e.g.* are policing workers also more likely to perform nursing duties?

(2) Are policing workers more likely to have activated ovaries than non-policing workers?

(3) Do policing and non-policing workers of the same age and patriline differ in the abundance of mRNA (transcriptomics (Cardoen *et al.* 2011)), peptides (peptidomics (Brockmann *et al.* 2009, Han *et al.* 2015)), proteins (proteomics (Cardoen *et al.* 2012)), biogenic amines (Reim and Scheiner 2014), or methylation status (epigenetics (Herb *et al.* 2012, Lockett *et al.* 2012)) in their brains?

(4) Do policing and non-policing workers of the same age and patriline differ in the levels of juvenile hormone (JH) (Schulz *et al.* 2002) or vitellogenin (Vg) (Nelson *et al.* 2007)? Juvenile hormone influences the transition from hive tasks to foraging (Robinson 2002) and might also influence other division of labor (reviewed in Johnson 2010). Vitellogenin interacts with juvenile hormone (Amdam and Omholt 2003, Guidugli *et al.* 2005, Wang *et al.* 2012) and has multiple effects on physiology, including task specialisation (Nelson *et al.* 2007).

Samples of policing and non-policing bees have been preserved at -80 °C, therefore some of the suggested studies (2, 3) aiming at unravelling the molecular signals that form a policing bee might be targeted by courageous students without too much delay. The determination of ovary developmental stage of frozen bees remains challenging (Inge Timmermans and Dries Cardoen, personal communication).

## 6.4 Other topics in worker policing

It has been suggested that policing behaviour varies through the seasons (Visscher 1996) and between subspecies (Kärcher and Ratnieks 2014). However, this has not yet been investigated. Comparisons in the speed of policing between several colonies each of *e.g.* Italian, Carniolan and dark bees (*A. m. ligustica, A. m. carnica, A. m. mellifera*), kept at the same apiaries, at several time points through the seasons of several years would be required- more effort than most people (and funding agencies) would be prepared to invest for such a small question.

## 6.5 Implications for human societies

It has been suggested that altruism in honeybee workers (*i.e.* foregoing of reproduction) is not voluntarily but enforced by mutual policing (Ratnieks and Wenseleers 2005, Wenseleers and Ratnieks 2006b, Ratnieks and Wenseleers 2008). Over evolutionary timescales, policing might have led to reproductive acquiescence, *i.e.* foregoing of attempts of reproduction ("self-policing") (Wenseleers *et al.* 2004a, Wenseleers *et al.* 2004b). Therefore, coercion (*e.g.* in the form of policing) is an important factor in social evolution in general (Frank 1995, Frank 1996, Frank 2003, Frank 2009, El Mouden *et al.* 2010, Bourke 2011, but see also Hauser *et al.* 2014). This also applies to vertebrate societies in the short term, for instance to pigtailed macaques (*Macaca nemestrina*), where policing stabilises social structures (Flack *et al.* 2005, Flack *et al.* 2006). Policing is also of importance to human societies (Kümmerli 2011). In fact, humans react strongly to (perceived) social control, *e.g.* to pairs of eyes (Figure 11), and in some cases even to symbols of eyes that are not necessarily recognized consciously (Figure 12) (Haley and Fessler 2005, Bateson *et al.* 2006, Burnham and Hare 2007, Rigdon *et al.* 2009, Francey and Bergmüller 2012, Nettle *et al.* 2012). However, reports by Fehr and Schneider 2010 and Raihani and Bshary 2012 suggest that the power of images to control behaviour is rather limited[31]. On the other hand, more direct forms of sanctions and punishment (which may be different from policing, because policing may be beneficial to the policing agent, whereas punishment is defined as costly (Clutton-Brock and Parker 1995, Raihani *et al*. 2012)) are a crucial part of contemporary human societies and likely have a long evolutionary history (e.g. West *et al*. 2011). Humans are

[31] This is supported by anecdotal evidence in the balance room of the research groups of Liliane Schoofs, Roger Huybrechts and Jozef Van den Broeck. A mutual not exclusive hypothesis is that many researchers are immune to social regulations and reputation, a notion that is further substantiated by additional observations on other occasions (personal observation).

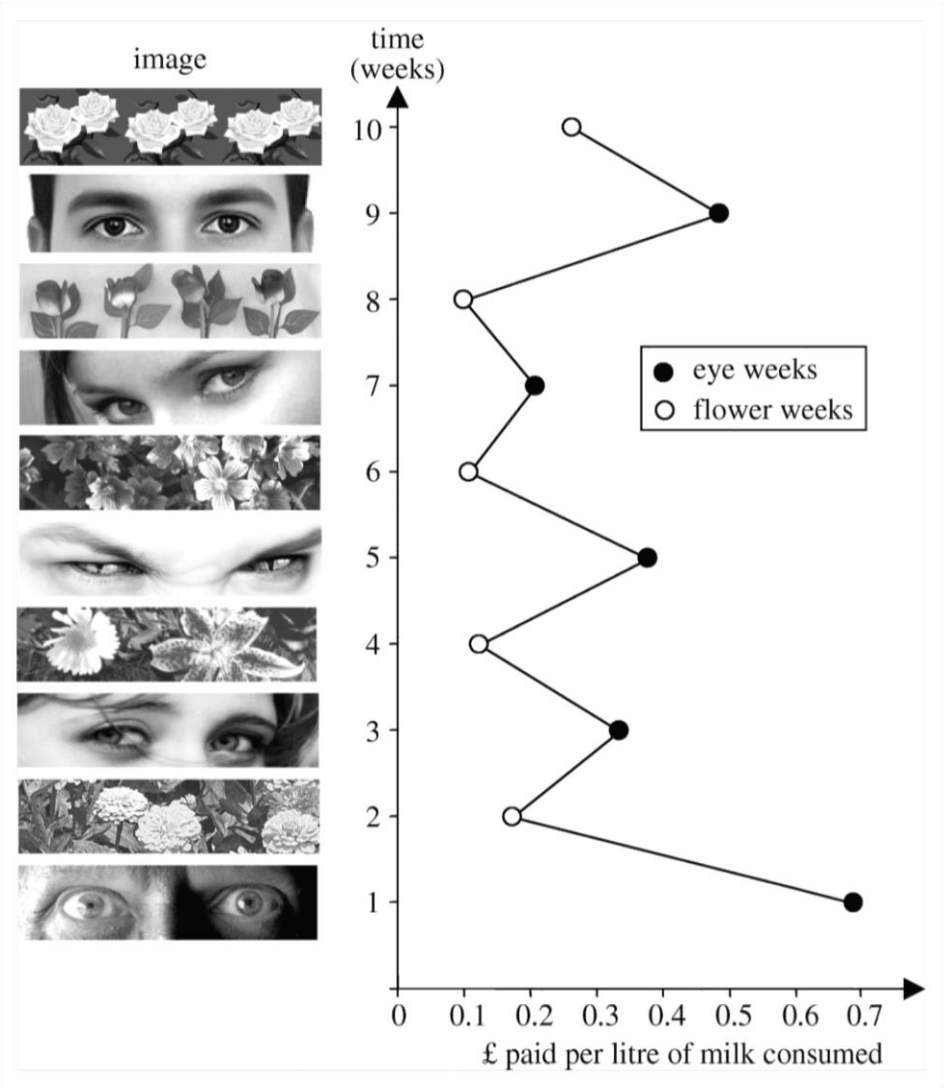


***Figure 11 – (Unconscious) perception of eyes can influence social behaviour.*** *In this experiment, members of an English psychology department paid 3 times more often for their drinks when perceiving cues of being watched (pictures of eyes). With kind permission of Bateson et al. 2006.*

keen to punish cheaters even if that incurs costs on them (Fehr and Gächter 2002, Hauert *et al.* 2007). Currently, the evolution of cooperation in general and in humans in particular is a vibrant field and receives attention from psychologists, social scientists, and biologists alike (Hauser *et al.* 2014). The vast literature on human cooperation is reviewed in Rand and Nowak 2013. To date it remains elusive whether policing has influenced the evolution of human altruism over and above the obvious immediate effects. Note that it is impossible to deduce any normative rules from observations and interpretations of behaviours of humans and other animals. In quite a different context and meaning, policing has become an important issue in science in general: namely the detection and punishment of unethical behaviour, be it plagiarism (Butler 2010), faking

and falsifying data (Goodman 2004), use and abuse of animals in research (Saunders 1989), or the ethics of clinical research on humans (Macilwain 2000). More policing would still be required in respect to ghost authorship and honorary (gift) authorship (Bogaert *et al.* 2009, Foundation *et al.* 2010, Wager and Kleinert 2011). Others suggest that prevention will be more efficient (Liliane Schoofs, personal communication). Likely, efficient policing is an effective deterrent.

***Figure 12 - Symbolic representations of faces (three dots on the left), but not other figures (three dots on the right) can be perceived as social control.*** *Modified, with kind permission of Rigdon et al. 2009.*

## 6.6 Epigenetics, ageing, and beekeeping

The discussion of the role of epigenetics in locusts (chapter 4, Epigenetics and locust life phase transitions) is close to exhaustive- it will be worthy to discuss this topic again once the suggested experiments (paragraph 4.8, Future research directions) will have been performed (some are being undertaken as I write these lines (Darron Cullen, personal communication; Eammon Mallon, personal communication)). Chen *et al.* 2015 showed that both father and mother are transmitting epigenetic information of phase state to their progeny, yet without further mechanistic elucidation. Robinson *et al.* 2015 report that in the migratory locust, *Locusta migratoria*, three key enzymes of the epigenetic machinery are differentially expressed in eggs of solitary and gregarious females. Epigenetics in social insects, however, will likely be one of the core themes of research in social insects in the next ten years (Welch and Lister 2014, Yan *et al.* 2014, Glastad *et al.* 2015, Simola *et al.* 2016).

The same applies to our discussion of current ageing research- we will have to wait for new research applying our suggestions to be published. In the meantime, time is passing to the advantage of our hypothesis and suggested experiments "[…] because its opponents eventually die" (Planck 1950).

The lately claimed connection between ageing and beekeeping in a study on positive effects of consuming bee products on telomere length (Nasir *et al.* 2015) is alas flawed on many levels and cannot be taken seriously.

While many problems in beekeeping have been attributed to pesticides, lack of flowers, pathogens and parasites, and a combination thereof, a substantial part of the problems may be prevented when beekeepers would be better educated. I argue that the transfer from scientific knowledge to practice is partly hindered by arrogance on the side of scientists and by an inefficient information transfer system and training scheme (or rather lack thereof) (Ernst *et al., Why are beekeepers reluctant to adjust their hive management to scientific insights?* (submitted to the Journal of Apicultural Research)). This need for education and training of beekeepers has been recognised in some countries, and in The Netherlands, a coordinating instance will be installed in October 2015 to promote bee health.

# 7 Summaries

## *7.1 Summary*

Policing is a form of controlling or suppressing unwanted behaviours of others. In social insects, policing is understood as the maintenance of a reproductive monopoly, which is mainly carried out by selective egg removal. Policing in the broader sense also occurs in the interaction within and between species of mammals, plants and bacteria.

Worker policing in the honeybee primarily occurs via selective removal of worker-laid eggs (WLE) but not queen-laid eggs (QLE). To date, it remains elusive how a bee could discriminate against WLE. We show that bee eggs are coated with over hundred peptide fragments of larger proteins. Many of these proteins have an antibiotic function and are also found in bee venom. Other proteins are linked to fertility. The observed quantitative differences in peptide abundancy between QLE and WLE are sufficient to discriminate against WLE.

Within the hive, particular honeybee workers specialise in policing behaviour: some workers remove many eggs, whereas other workers hardly or never do. Similarly, some patrilines (offspring of a particular father) participate not or less than expected in policing, whereas other patrilines remove many more eggs than expected based on the number of its members. This suggests that policing behaviour is heritable. There is no evidence for specialisation based on age.

In social insects, larval diet influences the development into distinct castes. Research into polyphenism and developmental plasticity has often relied on the caste phenomenon in social insects, which is under epigenetic control. Locusts undergo similar drastic changes when they start swarming, a behaviour that has been extensively studied for many years. Here, I discuss the evidence for the involvement of epigenetic mechanisms in the establishment and maintenance of the phase dimorphism between solitary and gregarious locusts. Similar to honeybees, it seems that epigenetics is crucial in the maintenance of a phase state, yet all evidence so far is observational and key genetic interventions are needed to provide causal evidence.

The honey bee is also a model organism to study ageing because of the pronounced difference in lifespan between queens, workers born in late summer, and other workers. For studies on lifespan in other organisms, we emphasize the importance of data obtained from wild or semi-wild populations where selection processes are stronger than in usually well maintained laboratory experiments where animals are provided with ample nutrients and shelter (chapter 5). Alternatively, stressors could be introduced in laboratory experiments.

## 7.2 Samenvatting[32]

Politiegedrag is een soort van controle of onderdrukking van ongewenst gedrag van anderen. Bij sociale insecten betekent politiegedrag het behoud van een voortplantingmonopolie, wat vooral door selectief verwijderen van eitjes gebeurt. Politiegedrag in de bredere zin komt ook voor binnen en tussen verschillende soorten van zoogdieren, planten en bacteriën.

Werkster politiegedrag bij de honingbij bestaat vooral in het selectieve verwijderen van eitjes gelegd door werksters (WLE), maar niet van eitjes gelegd door een koningin (QLE). Tot heden is niet gekend hoe werksters dit verschil kunnen maken. We tonen dat op het oppervlak van bijeneitjes meer dan honderd peptide fragmenten, afkomstig van grotere proteïnen, voorkomen. Veel van deze proteïnen hebben een antimicrobiele functie en worden ook in bijengif gevonden. Andere proteïnen zijn betrokken bij de vruchtbaarheid. De geobserveerde kwantitatieve verschillen tussen peptide hoeveelheid van QLE en WLE volstaan om de eitjes van werskster en de koningin te onderscheiden.

In een bijenkolonie zijn sommige werksters gespecialiseerd in politiegedrag: sommige verwijderen veel eitjes, andere daarentegen eten geen of nauwelijks eitjes. Soortgelijk doen sommige vaderlijnen (nageslacht van een bepaalde vader) niet of minder dan verwacht aan politiegedrag, andere vaderlijnen daarentegen verwijderen wel veel meer eitjes dan verwacht gebaseerd op het aantal leden van deze vaderlijnen. Dit suggereert dat politiegedrag erfelijk is. Er is geen bewijs gevonden voor specialisatie gebaseerd op leeftijd.

Bij sociale insecten beïnvloedt het voedsel dat larven opeten hun ontwikkeling tot verschillende kasten. Onderzoek naar polyfenisme en plasticiteit van ontwikkeling was vaak gebaseerd op dit kastenfenomeen dat onder epigenetische controle staat. Treksprinkhanen ondergaan gelijkaardige drastische veranderingen wanneer ze beginnen zwermen, een gedrag dat over vele jaren uitgebreid werd bestudeerd. Hier bespreek ik de aanwijzingen voor de betrokkenheid van epigenetische mechanismen bij het ontstaan en het behoud van fasepolymorfisme tussen solitaire en gregaire treksprinkhanen. Zoals bij honingbijen lijkt epigenetica noodzakelijk voor het behoud van een fase, maar tot nu toe zijn alle aanwijzingen slechts correlaties. Genetische ingrepen zijn nodig om causale verbanden aan te tonen.

De honingbij is ook een model organisme om veroudering te bestuderen dankzij de grote verschillen in levensduur tussen koninginnen, werksters geboren in de late zomer,

[32] A Dutch summary of this thesis.

en andere werksters. Voor onderzoek naar de levensduur van andere organismen beklemtonen we het belang van data bekomen van vrij levende of semi-vrij levende populaties waar de selectieprocessen onderworpen zijn aan meestal strengere natuurlijke omstandigheden dan die in optimale laboratorium-kweekomstandigheden. Alternatief zouden stress factoren geïntroduceerd worden in laboratoria proeven.

# 8 Acknowledgments

*I can thank these beautiful little creatures for many hours of the purest joy of discovery, interspersed among (to be sure) days and weeks of fruitless and sometimes discouraging work.*

Thomas D. Seeley, *Honeybee democracy*

I started the writing of my thesis with this very paragraph when having a little less then thirty-three and a third days left on my grant; as usual because I could not find the next word in another little piece of work that had nothing to do with my proper task. When I eventually will have succeeded in handing in this thesis, this is thanks to many people that kept supporting me and showed their interest in my work - even though some of them might at times have lost confidence that I would ever be finishing my PhD project.

I am obliged to my examination committee for their interest in my work and their support during the years, and especially for their time and expertise that helped to improve my thesis and manuscripts considerably.

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Many of the joys and sorrows of the PhD journey have been shared with Amélie and Wim, I am thankful for these supreme people.

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I appreciate the help of the mass spec folks Eisuke, Geert, Gerben, Kusay, Ruud, Sébastien, Wesley and the not only statistical advise of Jelle, Rik, and Wouter.

[33] „Behind every man (and woman) is a good secretary.“ (The late Hubert Markl on his 70th birthday)

I gratefully acknowledge the collaboration with Boris and Geert during my epigenetics detour which was challenging and pleasurable.

Several students have been working on parts of my dissertation. I hope they learned at least something, like dealing with difficult people, but for sure I can say that I on my part learned a lot from them. Thank you for your dedication: Alex, Alexander, Anna, Boris, Giel, Jolien, Nele, Sofie, Vincent.

Thanks are due to Fabian who created Figure 8 on short notice- this figure appeared on the cover of the Journal of Experimental Biology.

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I had the freedom to learn new techniques, acquire knowledge, come up with great experiments, excellent ideas, … and testing very few of them. My best invention I made in my second year: attaching a string to the door opening mechanism of the lab car so that one could open the cargo bay by pulling the string rather than having to crawl from the front to the back over all kind of material loaded (the door handle would not open the door from the outside); and fine tune the system by attaching the string to the sides of the car such that the string would not get trapped when slamming the door, and glide over any stuff loaded in the back of the car.

I was fortunate to meet quite some interesting people at conferences and meetings, and profited a lot from our interactions and their feedback, and I thank all these people for their encouragement.

I am grateful to have had the chance to interact with many fine scientists at the "Leuven Young Scientists and Beer" interdisciplinary journal club, which brought together the crème de la crème of international scientists to discuss flies, bees, brains, yeasts, and beers.

Whilst working towards this little booklet, I have been living at Kaboutermanstraat, Tiensesteenweg (2x), Albert-Giraud-Straat, Ruelensvest, Maria-Theresia-Straat, Predikherenstraat, Naamsestraat, Janseniusstraat, and for some time in Brighton and Gent, and I wish to thank my housemates for bearing my bad mood and never-ending complaints, and the many ways they found to distract me and lift my mood.

Parts of this thesis have (or should have) been written while I was a guest in Berlin, Gstaad, Singapore, Trondheim, and Uppsala, and I am indebted to my hosts for the opportunity to flee my office to focus on the writing part.

Over the years, most of my writings have been proof-read by family, friends and colleagues. Obviously I am fully responsible for all mistakes, misinterpretations, stylistic sins, spelling errors and other shortcomings, especially because I often did so against

better advice, but it is thanks to Aaron, Barbara, Claire, David, Heidi, Kate, Madeleine, and Paul that the level of errors is just about acceptable.

Finally, I thank most sincerely my family and friends for bearing (with) me. I can only imagine what a burden I must have been. I assume you will appreciate a thank you in person more than a few lines in a thesis, so I will do the former.

[34,35]

[34] Upper 1974, Molloy 1983, Skinner *et al.* 1985, Perry 1996, Skinner and Perlini 1996, Didden *et al.* 2007, Gardiner and Kearns 2011, Kearns and Gardiner 2011, Perry 2012, McLean and Thomas 2014

[35] „Schreiben geht entweder unendlich schnell oder unendlich langsam vor sich." [*Writing is either infinitely fast or infinitely slow.]* Friedrich Dürrenmatt, *Hingeschriebenes*

# 9 Varia

## 9.1 Curriculum Vitae

Uli Ernst was born in 1979 as the second son in a family of three multi-talented brothers. Growing up in Southern Germany, his childhood and adolescence were devoted to reading and sports (in that order), with some time spent on observing and rearing ants in his bedroom. He then studied Biology, Chemistry, and German Literature and Linguistics at the Universities of Konstanz and Tübingen. At the latter he became increasingly interested in Behavioural Ecology, and started beekeeping. After his Master's Thesis in Belfast and his graduation in Konstanz, he explored opportunities in the Netherlands and New Zealand. Finally, Uli decided to start his PhD adventure in Leuven in 2008, spent some time at the University of Sussex in the lab of the discoverer of policing, and succeeded in obtaining an IWT scholarship. The following years were filled with several successful (as well as unsuccessful) collaborations, more side than main projects, publications along the way, and the cumbersome compilation of this botch.

## 9.2 Grants & awards

IOCB PostDoctoral Scholarship, 2014
Best PhD award (PhD Interaction Day, KU Leuven), 2013
Best PhD student talk (19th Benelux Congress of Zoology), 2012
Best student talk (10th Hymenopterologen-Tagung), 2012
Best poster (NVG Netherlands Society for Behavioural Biology), 2010
IWT (Flemish agency for Innovation by Science and Technology) PhD scholarship, 2008-2012
DAAD (German Academic Exchange Service) scholarship for a Master's Thesis abroad (Belfast, UK), 2005

## 9.3 Publications & manuscripts

**Ernst, U.R.**, Cardoen, D., de Graaf, D.C., Mertens, I., Baggerman, G., Verleyen, P., Wenseleers, T., Schoofs, L. *Peptides mark the Difference between Eggs of Queens and Workers in honey bees.* [*i.e.* chapter 2; in preparation]

**Ernst, U.R.**, Cardoen, D., de Graaf, D.C., G., Verleyen, P., Wenseleers, T., Schoofs, L. *Specialisation on policing in the honeybee* Apis mellifera *L.* [*i.e.* chapter 3; in preparation]

**Ernst, U.R**., Detienne, G., Cardoen, D. *Why are beekeepers reluctant to adjust their hive management to scientific insights?* [submitted to the Journal of Apicultural Research]

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**Ernst, U.R**. (2016) *Inclusive Fitness 101: math not required*. Trends in Ecology and Evolution [in press]

**Ernst, U.** (2016) *Das Leben der Bienen.* Laborjournal [in press; in German]

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**Ernst, U.**, Detienne, G., De Haes, W., Schoofs, L., Temmerman, L. (2015) *Royalactine als verjongingskuur, ook voor de mens?* Maandblad van de Vlaamse Imkersbond, 101(2), 16-17 [in Dutch]

**Ernst, U**., Detienne, G. (2015). *Kein Wundermittel*. Deutsches Bienenjournal, 23(5), 58-59 [in German]

**Ernst, U.**, Detienne, G. (2015) *Das Königinnenenzym*. Deutsches Bienenjournal, 23(1), 51 [in German]

**Ernst, U.R.,** Depuydt, G., Van Hiel, M.B., Boerjan, B., De Loof, A., Schoofs, L. (2015) *Epigenetics and locust life phase transitions.* Journal of Experimental Biology, 218(1), 88-99 [*i.e.* chapter 4; cover story]

**Ernst, U.** (2014) *Demokratie zum Schwärmen.* Biologie in unserer Zeit, 44(5), 344 [in German]

**Ernst, U.R.** (2014) *Teaching and learning learning*. Trends in Ecology and Evolution, 29(12), 654

Detienne, G., De Haes, W, **Ernst, U.R**., Schoofs, L., Temmerman, L. (2014) *Royalactin mediates life-span extension in C. elegans via EGF-receptor*. Experimental Gerontology, 60, 129-135

**Ernst, U**. (2014) *Bienendemokratie.* Deutsches Bienenjournal, 22(3), 34 [in German]

**Ernst, U**., De Haes, W., Cardoen, D., Schoofs, L. (2014) *Life-prolonging measures for a dead theory?* AGE, 36(2), 533-534 [*i.e.* chapter 5]

Formesyn, E.M., Cardoen, D., **Ernst, U.R**., Danneels, E., Van Vaerenbergh, M., De Koker, D., Verleyen, P., Wenseleers, P., Schoofs, L., de Graaf, D.C. (2014) *Reproduction of honeybee workers is regulated by epidermal growth factor receptor signaling.* General and Comparative Endocrinology, 197, 1-4 [cover story]

Konijnendijk, N., Kern, A., Geeraerd, A., Topoloski, A., Smets, A., Souffreau, C., Dekeukeleire, D., Broeck, F. V. D., Galle, G., Roos, H., Heynen, H., Vos, I. D., Reniers, J., Pantel, J., Zweden, J. V., Coster, J. D., Boer, K. D., Spanier, K., Stevens, L., Morales, M. D. C. P., Fadil, N., Boon, N., Özdemir, S., Bracke, S., Scheepers, S., Meer, S. V. D., Huyse, T., **Ernst, U**. (2013). *Zogenaamde gevolgen van genderquota*. Campuskrant KU Leuven. Leuven [in Dutch]

**Ernst, U**. (2013) *Smells like hive spirit.* Trends in Ecology and Evolution, 28(12), 686-687

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**Ernst, U**. (2012) *Honeybee Democracy*. ISBE Newsletter, Supplement to Behavioral Ecology, 24(1), 8-9

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**Ernst, U**. (2011) *Inclusive fitness at all levels*. Trends in Ecology & Evolution, 26(9), 440

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## *9.4 Presentations*

Invited seminars

**Ernst, U.** (2014) *The Who and How of Worker Policing in the Honeybee*. University of Konstanz, Konstanz, Germany.

**Ernst, U.** (2013) Worker policing in the honeybee - how bees recognize and punish cheating (an interdisciplinary approach). PhD interaction day, KU Leuven, Leuven, Belgium. [36]

[36] Presentation generously held by Giel Detienne as I was not able to attend.

**Ernst, U**. (2013). *Ecophysiology of worker policing in the honeybee*. Ghent University, Ghent, Belgium.

**Ernst, U**. (2012). *Worker policing in the honeybee- why, who and how*. Université catholique de Louvain, Louvain-la-Neuve, Belgium.

**Ernst, U**. (2012). *Worker policing in the honeybee- who and how*. Royal Holloway University of London, London, UK.

Oral presentations

Detienne, G., De Haes, W., **Ernst, U**., Schoofs, L., Temmerman, L. (2015). *Fountain of youth for flies, worms and bees - effects and mode of action of royalactin*. Central European Meeting of Union for the Study of Social Insects (IUSSI). Lichtenfels, Germany.

Formesyn, E.M., Cardoen, D., **Ernst, U.R.**, Danneels, E., Van Vaerenbergh, M., De Koker, D., Verleyen, P., Wenseleers, P., Schoofs, L., de Graaf, D.C. (2013). *Fertility of honeybee workers is mediated by Epidermal Growth Factor Receptor (EGFR).* 60. Jahrestagung der Arbeitsgemeinschaft der Institute für Bienenforschung. Würzburg, Germany.

**Ernst, U**., Cardoen, D., Wenseleers, T., Verleyen, P., Schoofs, L. (2012). *The honeybee queen egg marking pheromone - a peptide?* 19th Benelux Congress of Zoology. Brussels, Belgium. [**best talk**]

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**Ernst, U**., Cardoen, D., Ratnieks, F., de Graaf, D., Schoofs, L., Verleyen, P., Wenseleers, T. (2011). *Hinweise auf Spezialisierung beim Arbeiter policing bei der Honigbiene* Apis mellifera. 58. Jahrestagung der Arbeitsgemeinschaft der Institute für Bienenforschung. Berlin, Germany.

**Ernst, U**., Cardoen, D., Ratnieks, F., Wenseleers, T., de Graaf, D., Schoofs, L., Verleyen, P. (2010). *Insights into worker policing in the honeybee* Apis mellifera. Annual Meeting of the Netherlands Society for Behavioural Biology. Soesterberg, The Netherlands.

**Ernst, U**., Cardoen, D., Ratnieks, F., de Graaf, D., Schoofs, L., Verleyen, P., Wenseleers, T. (2010). *Evidence for specialisation in worker policing in the honeybee* Apis mellifera*.* Bee-together Meeting. Ghent, Belgium.

**Ernst, U**., Cardoen, D., Verleyen, P., Wenseleers, T., de Graaf, D., Schoofs, L. (2008). *Worker policing in the honeybee* Apis mellifera *and other social insects.* Symposium "Entomology in Belgium 2008". Brussels, Belgium.

**Ernst, U**., Wenseleers, T., Verleyen, P., Cardoen, D., de Graaf, D., Schoofs, L., Ratnieks, F. (2008). *Allocation of worker policing in the honeybee* Apis mellifera. The 3rd European Conference of Apidology. Queen's University Belfast, Belfast, UK.

**Ernst, U**., Wenseleers, T., Verleyen, P., Cardoen, D., Schoofs, L., Ratnieks, F. (2008). *Worker policing in the honeybee- a job for a specialist?* 4th European Meeting of the International Union for the Study of Social Insects. La Roche-en-Ardenne, Belgium.

poster presentations

**Ernst, U**., Cardoen, D., Ratnieks, F., de Graaf, D., Schoofs, L., Verleyen, P., Wenseleers, T. (2012). *Spezialisierung auf "worker policing" bei der Honigbiene* Apis mellifera. 10. Hymenopterologen-Tagung. Stuttgart, Germany.

**Ernst, U**., Cardoen, D., Wenseleers, T., Verleyen, P., Schoofs, L. (2012). *Policing and peptides- how do honeybees recognize eggs?* 14th Congress of the International Society for Behavioral Ecology. Lund, Sweden.

Cardoen, D., **Ernst, U**., Schoofs, L., de Graaf, D., Wenseleers, T., Verleyen, P. (2010). Microarray analysis provides unprecedented insight into the detailed physiology of reproductive and non-reproductive honeybee workers. Bee-together Meeting. Ghent, Belgium.

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**Ernst, U**., Cardoen, D., Ratnieks, F., de Graaf, D., Schoofs, L., Verleyen, P., Wenseleers, T. (2010). *Is there specialisation in policing in the honeybee* Apis mellifera*?* IUSSI 2010. Copenhagen, Denmark.

Cardoen, D., **Ernst, U**., de Graaf, D., Wenseleers, T., Verleyen, P., Schoofs, L. (2010). *The altruistic infertility of honeybees: a physiological approach*. International Symposium in Honeybee Neuroscience. Berlin, Germany.

# 10 References

## 10.1 List of tables



## 10.2 List of figures

## *10.3 List of Supplementary Files*

Supplementary tables, figures, and files can be found online:
http://perswww.kuleuven.be/~u0058005/

### 10.3.1 Supplementary Tables

For Chapter 2 (Peptides mark the difference between eggs of queens and workers in honey bees):

**Supplementary Table 1 – QLE rinsed with water have higher removal rates than untreated QLE, but have similar hatchability**. This suggests that the higher removal rate was due to a (partial) removal of the queen egg marking pheromone (queen signal) and not due to damage or lower viability.

**Supplementary Table 2 – Identified peptides on QLE and WLE**. List of 518 peptides identified on QLE and WLE.

**Supplementary Table 3 – Peptides identified in queen's spermatheca**. A list of the 20 peptides identified in queen's spermatheca, with information on the occurrence on eggs.

**Supplementary Table 4 – Raw data for identified features.** The raw data for the 636 identified features, with information on abundancies, retention times, consensus sequence, and more details.

**Supplementary Table 5 – Abundancies for peptides**. The abundancies for 636 individual features are reported, together with the fold change between colonies and QLE vs. WLE.

**Supplementary Table 6 – Abundancies for consensus sequences**. The abundancies for 92 consensus sequences are reported, together with the fold change between colonies and QLE vs. WLE.

**Supplementary Table 7 – Abundancies for proteins**. The abundancies for 25 proteins are reported, together with the fold change between colonies and QLE vs. WLE.

**Supplementary Table 8 – PCA scores and factor loadings for protein, consensus, and peptide level.** In this file, the PCA scores for the 12 samples (6 QLE, 6 WLE) and the factor loadings for 25 proteins, 92 consensus sequences, and 636 individual features are reported.

### 10.3.2 Supplementary Figures

For Chapter 2 (Peptides mark the difference between eggs of queens and workers in honey bees):

**Supplementary Figure 1 –Biplot of Principal Component Analysis of consensus sequences**. Samples of the same hive cluster together. PC1 explains 49.8% of the variation and separates WLE (left) from QLE (right). PC2 explains 20.5% of the variation and separates colony 1 (upper part) from colony 2 (lower part).

**Supplementary Figure 2 - Biplot of Principal Component Analysis of peptides.** Samples of the same hive cluster together. PC1 explains 45.5 % of the variation and separates WLE (left) from QLE (right). PC2 explains 19.7 % of the variation and separates colony 1 (upper part) from colony 2 (lower part)

**Supplementary Figure 3 - Heatmap for abundancies of consensus sequences**. Most consensus sequences (overlapping peptides) are less abundant on QLE than on WLE. The samples cluster according to caste (QLE vs. WLE) and colony (colony 1 vs. colony 2). Relative abundancies are given as row z-scores.

**Supplementary Figure 4 - Heatmap for abundancies of peptides**. Most peptides are less abundant on QLE than on WLE. The samples cluster according to caste (QLE vs. WLE) and colony (colony 1 vs. colony 2). Relative abundancies are given as row z-scores.

**Supplementary Figure 5 – MA-plot for features, QLE vs. WLE**. The $log_2$-ratio of abundancies (on the level of features) between QLE and WLE is plotted against average intensity. Dashed lines indicate a twofold differences of abundancy in QLE (upper panel) and WLE (lower panel), respectively.

**Supplementary Figure 6 – Volcano-plot for features, QLE vs. WLE**. The negative decadic logarithm of adjusted p-values is plotted against the $log_2$-ratio of abundancies (on the level of features) between QLE and WLE. The horizontal dashed line indicates an adjusted p-value of 0.05. Vertical dashed lines indicate a twofold differences of abundancy in QLE (right panel) and WLE (left panel), respectively.

**Supplementary Figure 7 – MA-plot for features, colony 1 vs. colony 2**. The $log_2$-ratio of abundancies (on the level of features) between colony 1 and colony 2 is plotted against average intensity. Dashed lines indicate a twofold differences of abundancy in colony 1 (upper panel) and colony 2 (lower panel), respectively.

**Supplementary Figure 8 – Volcano-plot for features, colony 1 vs. colony 2**. The negative decadic logarithm of adjusted p-values is plotted against the $log_2$-ratio of abundancies (on the level of features) between colony 1 and colony 2. The horizontal dashed line indicates an adjusted p-value of 0.05. Vertical dashed lines indicate a twofold differences of abundancy in colony 1 (right panel) and colony 2 (left panel), respectively.

**Supplementary Figure 9 – MA-plot for consensus sequences, QLE vs. WLE**. The log2-ratio of abundancies (on the level of consensus sequences) between QLE and WLE is plotted against average intensity. Dashed lines indicate a twofold differences of abundancy in QLE (upper panel) and WLE (lower panel), respectively.

**Supplementary Figure 10 – Volcano-plot for consensus sequences, QLE vs. WLE**. The negative decadic logarithm of adjusted p-values is plotted against the $log_2$-ratio of abundancies (on the level of consensus sequences) between QLE and WLE. The horizontal dashed line indicates an adjusted p-value of 0.05. Vertical dashed lines indicate a twofold differences of abundancy in QLE (right panel) and WLE (left panel), respectively.

**Supplementary Figure 11 – MA-plot for consensus sequences, colony 1 vs. colony 2**. The $log_2$-ratio of abundancies (on the level of consensus sequences) between colony 1 and colony 2 is plotted against average intensity. Dashed lines indicate a twofold differences of abundancy in colony 1 (upper panel) and colony 2 (lower panel), respectively.

**Supplementary Figure 12 – Volcano-plot for consensus sequences, colony 1 vs. colony 2.** The negative decadic logarithm of adjusted p-values is plotted against the $log_2$-ratio of

abundancies (on the level of consensus sequences) between colony 1 and colony 2. The horizontal dashed line indicates an adjusted p-value of 0.05. Vertical dashed lines indicate a twofold differences of abundancy in colony 1 (right panel) and colony 2 (left panel), respectively.

**Supplementary Figure 13 – MA-plot for proteins, QLE vs. WLE**. The $log_2$-ratio of abundancies (on the level of proteins) between QLE and WLE is plotted against average intensity. Dashed lines indicate a twofold differences of abundancy in QLE (upper panel) and WLE (lower panel), respectively.

**Supplementary Figure 14 – Volcano-plot for proteins, QLE vs. WLE**. The negative decadic logarithm of adjusted p-values is plotted against the $log_2$-ratio of abundancies (on the level of proteins) between QLE and WLE. The horizontal dashed line indicates an adjusted p-value of 0.05. Vertical dashed lines indicate a twofold differences of abundancy in QLE (right panel) and WLE (left panel), respectively.

**Supplementary Figure 15 – MA-plot for proteins, colony 1 vs. colony 2**. The $log_2$-ratio of abundancies (on the level of proteins) between colony 1 and colony 2 is plotted against average intensity. Dashed lines indicate a twofold differences of abundancy in colony 1 (upper panel) and colony 2 (lower panel), respectively.

**Supplementary Figure 16 – Volcano-plot for proteins, colony 1 vs. colony 2**. The negative decadic logarithm of adjusted p-values is plotted against the $log_2$-ratio of abundancies (on the level of proteins) between colony 1 and colony 2. The horizontal dashed line indicates an adjusted p-value of 0.05. Vertical dashed lines indicate a twofold differences of abundancy in colony 1 (right panel) and colony 2 (left panel), respectively.

### 10.3.3 Supplementary Files

For Chapter 2 (Peptides mark the difference between eggs of queens and workers in honey bees):

**Supplementary File 1 – Additional information on proteins and potential routes to the egg surface**. In this document, additional information about the identified differential proteins and ways how these proteins may end up on eggs are provided.

For Chapter 4 (Epigenetics and locust life phase transitions):

**Supplementary File 2 – Overview of putative sequences of enzymes potentially involved in epigenetic regulation of gene expression in the migratory locust, *Locusta migratoria*.** BLAST searches against the genomes of *Apis mellifera* (Apm), *Drosophila melanogaster* (Drm), *Nasonia vitripennis* (Nsv), *Pediculus humanus corporis* (Peh), *Tribolium castaneum* (Trc) suggest the presence of at least six HDACs (histone deacetylase), two HATs (histone acetyl transferase), five HMTs (histone methyltransferase), two HDMs (histone demethylase), and one MBD (methyl binding protein) in *Locusta migratoria* (Lom).

**Supplementary File 3 - Putative sequences of enzymes potentially involved in epigenetic regulation of gene expression in the migratory locust, *Locusta migratoria***. A condensed view of Supplementary File 2, showing the genome sequence similarities between *Locusta migratoria* and several insect model organisms.

## *10.4 List of abbreviations*

| | |
|---|---|
| AKH | adipokinetic hormone |
| ANOVA | analysis of variance |
| APRP | adipokinetic hormone precursor-related peptide |
| Arg | arginine |
| AS | alternative splicing |
| Asp | aspartic acid |
| BCE | before the Common Era |
| BLAST | basic local alignment search tool |
| Cas | CRISPR-associated protein (→ CRISPR) |
| CC | *corpora cardiaca* |
| CCD | colony collapse disorder |
| CHC | cuticular hydrocarbon |
| ChIP-Seq | chromatin immunoprecipitation followed by sequencing of the immunoprecipitated DNA fragments (→ DNA) |
| CNS | central nervous system |
| Col | colony |
| CpG | cytosine followed by guanine |
| CpG O/E | ratio of observed vs. expected numbers of CpG dinucleotides (→ CpG) |
| CRISPR | clustered regularly interspaced short palindromic repeat |
| CSP | chemosensory protein |
| DG | diacyl-glycerols |
| DNA | deoxyribonucleic acid |
| Dnmt | DNA methyltransferase (→ DNA) |
| E/F | length of fore-wing/length of femur |
| EGFR | epidermal growth factor receptor |
| EST | expressed sequence tag |
| FC | fold change |
| F/C | length of femur/maximum head width |

| | |
|---|---|
| FDR | false discovery rate |
| GABA | γ-amino butyric acid |
| Gb | $10^9$ base-pairs |
| GLM | generalized linear model |
| GLMM | generalized linear mixed model |
| Glu | glutamic acid |
| Gly | glycine |
| GPCR | G-protein coupled receptor |
| H3K27ac | acetylated lysine 27 in histone 3 |
| HAT | histone acetyl transferase |
| HDAC | histone deacetylase |
| HDACi | histone deacetylase inhibitor |
| HDM | histone demethylase |
| HMT | histone methyltransferase |
| $^1$H NMR | proton nuclear magnetic resonance |
| HPLC-GC/MS | high performance liquid chromatography- gas chromatography coupled mass spectrometry |
| HSP | heat-shock protein |
| 5-HTR | serotonin (=5-hydroxytryptamine) receptor |
| JH | juvenile hormone |
| JHPH | juvenile hormone binding protein, hexamerins, prophenoloxidase, and hemocyanins |
| LITE | light-inducible transcriptional effector |
| LMPP | *Locusta migratoria* pacifastin-related precursor |
| lncRNA | long noncoding RNA (→ RNA) |
| Lom | *Locusta migratoria* |
| Lom-OMP | *Locusta migratoria* ovary maturating parsin |
| MBD | methyl binding protein |
| $M_e$ | effective paternity |
| miRNA | microRNA (→ RNA) |
| ncRNA | non-coding RNA (→ RNA) |
| NDE | non-detection error |
| NP-A | neuroparsin A |
| NPP | neuroparsin precursors |
| NSE | non-sampling error |

| | |
|---|---|
| nt | nucleotide(s) |
| OMP | ovary maturating parsin |
| OSTA | oxidative stress theory of aging |
| PCA | principal component analysis |
| piRNA | PIWI-interacting RNA (→ PIWI, → RNA) |
| PE | phosphatidylethanol-amines |
| PIWI | P-element induced wimpy testis |
| PRP | phase-related factor |
| PTM | posttranslational modifications |
| QLE | queen-laid egg(s) |
| QMP | queen mandibular pheromone |
| rcf | relative centrifugal force |
| rDNA | ribosomal DNA (→ DNA) |
| RNA | ribonucleic acid |
| RNAi | RNA interference (→ RNA) |
| ROS | reactive oxygen species |
| RRBS | reduced representational bisulfite sequencing |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| Scg | *Schistocerca gregaria* |
| sd | standard deviation |
| SEM | scanning electron microscopy |
| siRNA | short interfering RNA (→ RNA) |
| SGPP | *Schistocerca gregaria* pacifastin-like precursor |
| TALE | transcription activator-like effector |
| TALEN | transcription activator-like effector nuclease |
| TCD | tritocerebral dwarf |
| TCG | tritocerebral giant |
| TEM | transmission electron microscopy |
| TET | ten-eleven translocation |
| Vg | vitellogenin |
| WGBS | whole genome bisulfite sequencing |
| WLE | worker-laid egg(s) |
| YP | yellow protein |
| ZFN | zinc finger nuclease |

## *10.5 Bibliography*


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